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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
14 December 2000 (14.12.2000)

PCT

(10) International Publication Number  
**WO 00/75163 A1**

- (51) International Patent Classification<sup>7</sup>: C07H 21/04, (74) Agents: WALES, Michele, M. et al.; Human Genome Sciences, Inc., 9410 Key West Avenue, Rockville, MD 20850 (US).  
C12N 15/63
- (21) International Application Number: PCT/US00/14925
- (22) International Filing Date: 1 June 2000 (01.06.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 60/137,796 3 June 1999 (03.06.1999) US
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- (81) Designated States (national): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
- With international search report.
  - Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 00/75163 A1

(54) Title: ANGIOGENIC PROTEINS AND USES THEREOF

(57) Abstract: Disclosed are human angiogenic polypeptides, biologically active, diagnostically or therapeutically useful fragments, analogs, or derivatives thereof, and DNA(RNA) encoding such angiogenic polypeptides. Also provided are procedures for producing such polypeptides by recombinant techniques and antibodies, agonists and antagonists against such polypeptides. Such polypeptides and polynucleotides may be used therapeutically for stimulating wound healing and for vascular tissue repair. Also provided are methods of using the antibodies and antagonists to inhibit tumor angiogenesis and thus tumor growth, inflammation, diabetic retinopathy, rheumatoid arthritis, and psoriasis. Further provided are methods of using the antibodies and agonists to promote angiogenesis and lymphangiogenesis. The antagonists may also be used to treat chronic inflammation caused by increased vascular permeability. In addition to these disorders, the antagonists may also be employed to treat retinopathy associated with diabetes, rheumatoid arthritis and psoriasis. The agonists and/or antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

## Angiogenic Proteins and Uses Thereof

### Background of the Invention

#### Field of the Invention

5       The present invention relates to angiogenic proteins, and agonists or antagonists of angiogenic proteins. The invention further relates to screening methods for identifying agonists and antagonists of angiogenic protein activities.

#### Related Art

10       The formation of new blood vessels, or angiogenesis, is essential for embryonic development, subsequent growth, and tissue repair. Angiogenesis is also an essential part of certain pathological conditions, such as neoplasia (*i.e.*, tumors and gliomas). Abnormal angiogenesis is associated with other diseases such as inflammation, rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman, J. and Klagsbrun, M.,  
15   *Science* 235:442-447(1987)).

      Both acidic and basic fibroblast growth factor molecules are mitogens for endothelial cells and other cell types. Angiotropin and angiogenin can induce angiogenesis, although their functions are unclear (Folkman, J., *Cancer Medicine*, Lea and Febiger Press, pp. 153-170 (1993)). A highly selective mitogen for vascular  
20   endothelial cells is vascular endothelial growth factor or VEGF (Ferrara, N. *et al.*, *Endocr. Rev.* 13:19-32 (1992)), which is also known as vascular permeability factor (VPF).

      Vascular endothelial growth factor is a secreted angiogenic mitogen whose target cell specificity appears to be restricted to vascular endothelial cells. The murine VEGF  
25   gene has been characterized and its expression pattern in embryogenesis has been analyzed. A persistent expression of VEGF was observed in epithelial cells adjacent to fenestrated endothelium, *e.g.*, in choroid plexus and kidney glomeruli. The data was consistent with a role of VEGF as a multifunctional regulator of endothelial cell growth and differentiation (Breier, G. *et al.*, *Development* 114:521-532 (1992)).

30       VEGF shares sequence homology with human platelet-derived growth factors, PDGFa and PDGFb (Leung, D.W., *et al.*, *Science* 246:1306-1309, (1989)). The extent of homology is about 21% and 23%, respectively. Eight cysteine residues contributing to disulfide-bond formation are strictly conserved in these proteins. Although they are similar, there are specific differences between VEGF and PDGF. While PDGF is a  
35   major growth factor for connective tissue, VEGF is highly specific for endothelial cells. Alternatively spliced mRNAs have been identified for both VEGF, PLGF, and PDGF and these different splicing products differ in biological activity and in receptor-binding

specificity. VEGF and PDGF function as homo-dimers or hetero-dimers and bind to receptors which elicit intrinsic tyrosine kinase activity following receptor dimerization.

VEGF has four different forms of 121, 165, 189 and 206 amino acids due to alternative splicing. VEGF121 and VEGF165 are soluble and are capable of promoting angiogenesis, whereas VEGF189 and VEGF206 are bound to heparin containing proteoglycans in the cell surface. The temporal and spatial expression of VEGF has been correlated with physiological proliferation of the blood vessels (Gajdusek, C.M., and Carbon, S.J., *Cell Physiol.* 139:570-579 (1989); McNeil, P.L., *et al.*, *J. Cell. Biol.* 109:811-822 (1989)). Its high affinity binding sites are localized only on endothelial cells in tissue sections (Jakeman, L.B., *et al.*, *Clin. Invest.* 89:244-253 (1989)). The factor can be isolated from pituitary cells and several tumor cell lines, and has been implicated in some human gliomas (Plate, K.H., *Nature* 359:845-848 (1992)). Interestingly, expression of VEGF121 or VEGF165 confers on Chinese hamster ovary cells the ability to form tumors in nude mice (Ferrara, N. *et al.*, *J. Clin. Invest.* 91:160-170 (1993)). The inhibition of VEGF function by anti-VEGF monoclonal antibodies was shown to inhibit tumor growth in immune-deficient mice (Kim, K.J., *Nature* 362:841-844 (1993)). Further, a dominant-negative mutant of the VEGF receptor has been shown to inhibit growth of glioblastomas in mice.

Vascular permeability factor (VPF) has also been found to be responsible for persistent microvascular hyperpermeability to plasma proteins even after the cessation of injury, which is a characteristic feature of normal wound healing. This suggests that VPF is an important factor in wound healing. Brown, L.F. *et al.*, *J. Exp. Med.* 176:1375-1379 (1992).

The expression of VEGF is high in vascularized tissues, (*e.g.*, lung, heart, placenta and solid tumors) and correlates with angiogenesis both temporally and spatially. VEGF has also been shown to induce angiogenesis *in vivo*. Since angiogenesis is essential for the repair of normal tissues, especially vascular tissues, VEGF has been proposed for use in promoting vascular tissue repair (*e.g.*, in atherosclerosis).

U.S. Patent No. 5,073,492, issued December 17, 1991 to Chen *et al.*, discloses a method for synergistically enhancing endothelial cell growth in an appropriate environment which comprises adding to the environment, VEGF, effectors and serum-derived factor. Also, vascular endothelial cell growth factor C sub-unit DNA has been prepared by polymerase chain reaction techniques. The DNA encodes a protein that may exist as either a heterodimer or homodimer. The protein is a mammalian vascular endothelial cell mitogen and, as such, is useful for the promotion of vascular development and repair, as disclosed in European Patent Application No. 92302750.2, published September 30, 1992.



Recent additions to the vascular endothelial growth factor family include VEGF-3, VEGF-D, and VEGF-E. VEGF-3 growth factors are strong mitogens which promote the proliferation of vascular endothelial and/or mesodermal cells, as disclosed in International Publication No. WO 96/39421, published Dec. 12, 1996 to Hu et al.

5 VEGF-D is another member of the vascular endothelial growth factor family, and has the ability to stimulate and/or enhance the proliferation or differentiation of endothelial cells, as disclosed in International Publication No. WO 98/07832, published February 26, 1998. More recently, Meyer et al. published a new member of the vascular endothelial growth factor family, naming it VEGF-E. VEGF-E is a potent angiogenic factor that  
10 selectively binds to VEGF receptor-2 (KDR) (Meyer *et al.*, *EMBO J*, 18:363-374 (1999)).

Thus, there is a need for treatments which promote or inhibit angiogenic proteins.

### *Summary of the Invention*

15 The present invention relates to members of the VEGF/PDGF families of proteins, and related proteins, collectively referred to herein as "angiogenic proteins" or "angiogenic polypeptides", as well as agonists and/or antagonists of angiogenic proteins.

In accordance with a further aspect of the present invention, there are provided  
20 processes for utilizing such polypeptides or polynucleotides encoding such polypeptides for therapeutic purposes, for example, to stimulate angiogenesis, wound-healing, growth of damaged bone and tissue, and to promote vascular tissue repair. In particular, there are provided processes for utilizing such polypeptides, or polynucleotides encoding such polypeptides, for treatment of peripheral artery disease, such as critical limb ischemia and  
25 coronary disease. In accordance with yet another aspect of the present invention, there are provided agonists to such polypeptides, which may be used to enhance the action of such polypeptides, for example, to promote angiogenesis, lymphangiogenesis, or to promote vascular tissue repair. The present invention further relates to variants of the hereinabove described polypeptides which encode for antigenic or immunogenic epitopes of the  
30 polypeptides.

In accordance with yet another aspect of the present invention, there are provided agonists to such polypeptides, which may be used to enhance or promote the action of such polypeptides, for example, to prevent tumor angiogenesis and thus inhibit the growth of tumors, to treat diabetic retinopathy, inflammation, rheumatoid arthritis and  
35 psoriasis. In accordance with yet another aspect of the present invention, there are provided agonists to such polypeptides, which may be used to enhance the action of such

polypeptides, for example, to promote angiogenesis, lymphangiogenesis, or to promote vascular tissue repair.

In accordance with yet another aspect of the present invention, there are provided antibodies against such polypeptides and processes for producing such antibodies. In accordance with yet a further aspect of the present invention, there are provided processes for utilizing such antibodies for therapeutic purposes, for example, to stimulate angiogenesis, wound-healing, growth of damaged bone and tissue, to promote vascular tissue repair, and to stimulate lymphangiogenesis. In particular, there are provided processes for utilizing such antibodies for treatment of peripheral artery disease, such as critical limb ischemia and coronary disease.

In accordance with yet another aspect of the present invention, there are provided antagonists to such polypeptides, which may be used to inhibit the action of such polypeptides, for example, to prevent tumor angiogenesis and thus inhibit the growth of tumors, to treat diabetic retinopathy, inflammation, rheumatoid arthritis and psoriasis.

In accordance with one aspect of the present invention, there are provided novel mature polypeptides, as well as biologically active and diagnostically or therapeutically useful fragments, analogs, and derivatives thereof. The polypeptides of the present invention are of human origin.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules comprising polynucleotides encoding full length or truncated angiogenic polypeptides having the amino acid sequences shown in Table 1. The present invention also relates to biologically active and diagnostically or therapeutically useful fragments, analogs, and derivatives of angiogenic proteins.

In accordance with still another aspect of the present invention, there are provided processes for producing such polypeptides by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding a polypeptide of the present invention, under conditions promoting expression of said proteins and subsequent recovery of said proteins.

In accordance with another aspect of the present invention, there are provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to nucleic acid sequences of the present invention. In accordance with another aspect of the present invention, there are provided methods of diagnosing diseases or a susceptibility to diseases related to mutations in nucleic acid sequences of the present invention and proteins encoded by such nucleic acid sequences.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides,

for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors.

In accordance with yet another aspect of this invention, there are provided methods of screening for agonists and/or antagonists of angiogenic proteins, that may enhance or inhibit the desired activity.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

### ***Brief Description of the Figures***

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figures 1A-1E show the full length nucleotide (SEQ ID NO:15) and the deduced amino acid (SEQ ID NO:16) sequence of VEGF2. The polypeptide comprises approximately 419 amino acid residues of which approximately 23 represent the leader sequence. The standard one letter abbreviations for amino acids are used. Sequencing was performed using the Model 373 Automated DNA Sequencer (Applied Biosystems, Inc.). Sequencing accuracy is predicted to be greater than 97%.

Figures 2A-2D show the nucleotide (SEQ ID NO:25) and the deduced amino acid (SEQ ID NO:26) sequence of a truncated, biologically active form of VEGF2. The polypeptide comprises approximately 350 amino acid residues of which approximately the first 24 amino acids represent the leader sequence.

Figures 3A-3C are an illustration of the amino acid sequence homology between PDGF-A (SEQ ID NO: 2), PDGF-B (SEQ ID NO: 4), PIGF (SEQ ID NO: 6), PIGF-2 (SEQ ID NO: 8), GDGF (SEQ ID NO: 10), VEGF (SEQ ID NO: 12), VEGF-A (SEQ ID NO: 14), VEGF-2 (SEQ ID NO: 16), VEGF-3 (SEQ ID NO: 18), VEGF-D (SEQ ID NO: 20), VEGF-D1 (SEQ ID NO: 22), and VEGF-E (SEQ ID NO: 24). The boxed areas indicate the conserved sequences and the location of the eight conserved cysteine residues.

### ***Detailed Description of the Preferred Embodiments***

#### ***Definitions***

In accordance with one aspect of the present invention, there are provided polypeptide molecules comprising members of the PDGF and VEGF families. More specifically, the members of the PDGF and VEGF families include: Platelet Derived Growth Factor-A (PDGF-A), as disclosed in European Patent Number EP-682110 (SEQ ID NO: 1-2); Platelet Derived Growth Factor-B (PDGF-B), as disclosed in European

Patent Number EP-282317 (SEQ ID NO: 3-4); Placental Growth Factor (PIGF), as disclosed in International Publication Number WO 92/06194 (SEQ ID NO: 5-6); Placental Growth Factor-2 (PIGF-2), as disclosed in Hauser *et al.*, *Growth Factors*, 4:259-268 (1993) (SEQ ID NO: 7-8); Glioma Derived Growth Factor (GDGF), as disclosed in European Patent Number EP-399816 (SEQ ID NO: 9-10); Vascular Endothelial Growth Factor (VEGF), as disclosed in International Publication Number WO 90/13649 (SEQ ID NO: 11-12); Vascular Endothelial Growth Factor-A (VEGF-A), as disclosed in European Patent Number EP-506477 (SEQ ID NO: 13-14); Vascular Endothelial Growth Factor-2 (VEGF-2), as disclosed in International Publication Number WO 96/39515 (SEQ ID NO: 15-16); Vascular Endothelial Growth Factor-3 (VEGF-3), as disclosed in International Publication Number WO 96/39421 (SEQ ID NO: 17-18); Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/07832 (SEQ ID NO: 19-20); Vascular Endothelial Growth Factor-D1 (VEGF-D1), as disclosed in International Publication Number WO 98/02543 (SEQ ID NO: 21-22); and Vascular Endothelial Growth Factor-E (VEGF-E), as disclosed in German Patent Number DE19639601 (SEQ ID NO: 23-24), and are collectively referred herein as "angiogenic proteins" or "angiogenic polypeptides". The above mentioned references are herein incorporated by reference in their entirety.

As used herein, "agonist" refers to any polynucleotide, polypeptide, antibody, or molecule, including small molecules, that promotes or enhances the desired activity of the angiogenic proteins.

As used herein, "antagonist" refers to any polynucleotide, polypeptide, antibody, or molecule, including small molecules, that diminishes or inhibits the desired activity of the angiogenic proteins.

As used herein, "desired activity" refers to the ability of agonists and/or antagonists to promote endothelial cell proliferation (angiogenesis or lymphangiogenesis), or to inhibit endothelial cell proliferation (angiogenesis or lymphangiogenesis) in relation to the natural activity of angiogenic proteins.

### ***Agonists and Antagonists of Angiogenic Proteins***

The present invention relates to agonists and/or antagonists of angiogenic proteins.

One embodiment of angiogenic protein agonists includes antibodies which binds to the polypeptide and promotes or enhances the desired activity. Examples of these agonists include monoclonal or polyclonal antibodies directed to specific angiogenic polypeptides which bind the angiogenic polypeptides and promote the angiogenic activity of those polypeptides, for example, by stabilizing the dimerized form of the angiogenic

protein, or by aggregating the polypeptides that are bound to the antibodies, and thereby concentrating or localizing the activity of the angiogenic polypeptides.

Another potential angiogenic protein agonist include small molecules that promote or enhance the activity of angiogenic proteins. Such small molecule agonists may be  
5 involved in binding to angiogenic protein receptors, for example, or related receptors, and thereby enhance the activities of angiogenic proteins.

In a further embodiment, angiogenic protein agonists include polypeptides that enhance the activity of angiogenic proteins. Such polypeptide agonists may be involved in binding to angiogenic proteins, for example, or related receptors, and thereby enhance  
10 the activities of angiogenic proteins.

Another example includes antisense constructs that promote or enhance the desired activity of the angiogenic proteins.

Potential angiogenic protein antagonists include an antibody, or in some cases, an oligonucleotide, which binds to the polypeptide and effectively eliminate angiogenic  
15 protein activity. Alternatively, a potential antagonist may be a closely related protein which binds to angiogenic protein receptors, however, they are inactive forms of the polypeptide and thereby prevent the action of angiogenic proteins. Examples of these antagonists include a negative dominant mutant of an angiogenic protein polypeptide, for example, one chain of a hetero-dimeric form of an angiogenic protein may be dominant  
20 and may be mutated such that biological activity is not retained. An example of a negative dominant mutant includes truncated versions of a dimeric angiogenic protein which is capable of interacting with another dimer to form wild type angiogenic protein, however, the resulting homo-dimer is inactive and fails to exhibit characteristic desired activity.

Another potential angiogenic protein antagonist is an antisense construct prepared  
25 using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10  
30 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee *et al.*, *Nucl. Acids Res.* 6:3073 (1979); Cooney *et al.*, *Science* 241:456 (1988); and Dervan *et al.*, *Science* 251:1360 (1991)), thereby preventing transcription and the production of an angiogenic protein. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks  
35 translation of the mRNA molecule into the angiogenic protein polypeptide (Antisense - Okano, *J. Neurochem.* 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described

above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the angiogenic protein.

In a further embodiment, angiogenic protein antagonists also include small molecules which bind to and occupy the active site of the polypeptide thereby making the catalytic site inaccessible to substrate such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The antagonists may be employed to limit angiogenesis necessary for solid tumor metastasis. Since angiogenesis and neovascularization are essential steps in solid tumor growth, inhibition of angiogenic activity of the angiogenic protein is very useful to prevent the further growth, retard, or even regress solid tumors.

This invention is also related to a method of screening compounds to identify those which are angiogenic protein agonists or antagonists. An example of such a method takes advantage of the ability of angiogenic proteins to significantly stimulate the proliferation of human endothelial cells in the presence of the mitogen Con A. Endothelial cells are obtained and cultured in 96-well flat-bottomed culture plates (Costar, Cambridge, MA) in a reaction mixture supplemented with Con-A (Calbiochem, La Jolla, CA). Con-A, polypeptides of the present invention and the compound to be screened are added. After incubation at 37°C, cultures are pulsed with 1 FCi of  $^3\text{H}$ thymidine (5 Ci/mmol; 1 Ci = 37 BGq; NEN) for a sufficient time to incorporate the  $^3\text{H}$  and harvested onto glass fiber filters (Cambridge Technology, Watertown, MA). Mean  $^3\text{H}$ -thymidine incorporation (cpm) of triplicate cultures is determined using a liquid scintillation counter (Beckman Instruments, Irvine, CA). Significant  $^3\text{H}$ thymidine incorporation, as compared to a control assay where the compound is excluded, indicates stimulation of endothelial cell proliferation.

To assay for agonists, the assay described above is performed and the ability of the compound to promote  $^3\text{H}$ thymidine incorporation in the presence of an angiogenic protein indicates that the compound is an agonist to the angiogenic protein. Conversely, to assay for antagonists, the assay described above is performed and the ability of the compound to inhibit  $^3\text{H}$ thymidine incorporation in the presence of an angiogenic protein indicates that the compound is an antagonist to the angiogenic protein. Alternatively, antagonists to an angiogenic protein may be detected by combining an angiogenic protein and a potential antagonist with membrane-bound angiogenic protein receptors or recombinant receptors under appropriate conditions for a competitive inhibition assay. The angiogenic protein can be labeled, such as by radioactivity, such that the number of angiogenic protein molecules bound to the receptor can determine the effectiveness of the potential antagonist.

Alternatively, the response of a known second messenger system following interaction of the angiogenic protein and receptor would be measured and compared in the presence or absence of the compound. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis. In another method, a mammalian cell or membrane preparation expressing the angiogenic protein receptor is incubated with labeled angiogenic protein in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured.

### Epitopes and Antibodies

The present invention encompasses polypeptides comprising, or alternatively consisting of, an epitope of the polypeptide having an amino acid sequence of SEQ ID NOS:2 or 4, or an epitope of the polypeptide sequence encoded by a polynucleotide sequence contained in ATCC Deposit No: 97149 or 75698 or encoded by a polynucleotide that hybridizes to the complement of the sequence of SEQ ID NOS: 1 or 3 or contained in ATCC Deposit No: 97149 or 75698 under stringent hybridization conditions or lower stringency hybridization conditions as defined *supra*. A representative clone containing the sequence for SEQ ID NO:2 or 4, was deposited with the American Type Culture Collection ("ATCC") on March 4, 1994, and given ATCC Deposit Number 75698, and on May 12, 1995, and given ATCC Deposit Number 97149. The ATCC is located at 10801 University Boulevard, Manassas, Virginia 20110-2209, USA. The ATCC deposit was made pursuant to the terms of the Budapest Treaty on the international recognition. The present invention further encompasses polynucleotide sequences encoding an epitope of a polypeptide sequence of the invention (such as, for example, the sequence disclosed in SEQ ID NOS: 1 or 3), polynucleotide sequences of the complementary strand of a polynucleotide sequence encoding an epitope of the invention, and polynucleotide sequences which hybridize to the complementary strand under stringent hybridization conditions or lower stringency hybridization conditions defined *supra*.

The term "epitopes," as used herein, refers to portions of a polypeptide having antigenic or immunogenic activity in an animal, preferably a mammal, and most preferably in a human. In a preferred embodiment, the present invention encompasses a polypeptide comprising an epitope, as well as the polynucleotide encoding this polypeptide. An "immunogenic epitope," as used herein, is defined as a portion of a protein that elicits an antibody response in an animal, as determined by any method known in the art, for example, by the methods for generating antibodies described *infra*.

(See, for example, Geysen et al., Proc. Natl. Acad. Sci. USA 81:3998-4002 (1983)).

The term "antigenic epitope," as used herein, is defined as a portion of a protein to which an antibody can immunospecifically bind its antigen as determined by any method well known in the art, for example, by the immunoassays described herein. Immunospecific  
5 binding excludes non-specific binding but does not necessarily exclude cross-reactivity with other antigens. Antigenic epitopes need not necessarily be immunogenic.

Fragments which function as epitopes may be produced by any conventional means. (See, e.g., Houghten, Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985), further described in U.S. Patent No. 4,631,211).

10 In the present invention, antigenic epitopes preferably contain a sequence of at least 4, at least 5, at least 6, at least 7, more preferably at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, and, most preferably, between about 15 to about 30 amino acids. Preferred polypeptides comprising immunogenic or antigenic epitopes are at least 10, 15,  
15 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amino acid residues in length. Additional non-exclusive preferred antigenic epitopes include the antigenic epitopes disclosed herein, as well as portions thereof. Antigenic epitopes are useful, for example, to raise antibodies, including monoclonal antibodies, that specifically bind the epitope. Preferred antigenic epitopes include the antigenic epitopes  
20 disclosed herein, as well as any combination of two, three, four, five or more of these antigenic epitopes. Antigenic epitopes can be used as the target molecules in immunoassays. (See, for instance, Wilson et al., Cell 37:767-778 (1984); Sutcliffe et al., Science 219:660-666 (1983)).

Similarly, immunogenic epitopes can be used, for example, to induce antibodies  
25 according to methods well known in the art. (See, for instance, Sutcliffe et al., *supra*; Wilson et al., *supra*; Chow et al., Proc. Natl. Acad. Sci. USA 82:910-914; and Bittle et al., J. Gen. Virol. 66:2347-2354 (1985)). Preferred immunogenic epitopes include the immunogenic epitopes disclosed herein, as well as any combination of two, three, four, five or more of these immunogenic epitopes. The polypeptides comprising one or more  
30 immunogenic epitopes may be presented for eliciting an antibody response together with a carrier protein, such as an albumin, to an animal system (such as rabbit or mouse), or, if the polypeptide is of sufficient length (at least about 25 amino acids), the polypeptide may be presented without a carrier. However, immunogenic epitopes comprising as few  
35 as 8 to 10 amino acids have been shown to be sufficient to raise antibodies capable of binding to, at the very least, linear epitopes in a denatured polypeptide (e.g., in Western blotting).



Epitope-bearing polypeptides of the present invention may be used to induce antibodies according to methods well known in the art including, but not limited to, in vivo immunization, in vitro immunization, and phage display methods. See, e.g., Sutcliffe et al., *supra*; Wilson et al., *supra*, and Bittle et al., *J. Gen. Virol.*, 66:2347-2354 (1985). If in vivo immunization is used, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling the peptide to a macromolecular carrier, such as keyhole limpet hemacyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine residues may be coupled to a carrier using a linker such as maleimidobenzoyl- N-hydroxysuccinimide ester (MBS), while other peptides may be coupled to carriers using a more general linking agent such as glutaraldehyde. Animals such as rabbits, rats and mice are immunized with either free or carrier- coupled peptides, for instance, by intraperitoneal and/or intradermal injection of emulsions containing about 100  $\mu$ g of peptide or carrier protein and Freund's adjuvant or any other adjuvant known for stimulating an immune response. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody which can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies according to methods well known in the art.

As one of skill in the art will appreciate, and as discussed above, the polypeptides of the present invention comprising an immunogenic or antigenic epitope can be fused to other polypeptide sequences. For example, the polypeptides of the present invention may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, or any combination thereof and portions thereof), resulting in chimeric polypeptides. Such fusion proteins may facilitate purification and may increase half-life in vivo. This has been shown for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. See, e.g., EP 394,827; Trauneker et al., *Nature*, 331:84-86 (1988). Enhanced delivery of an antigen across the epithelial barrier to the immune system has been demonstrated for antigens (e.g., insulin) conjugated to an FcRn binding partner such as IgG or Fc fragments (see, e.g., PCT Publications WO 96/22024 and WO 99/04813). IgG Fusion proteins that have a disulfide-linked dimeric structure due to the IgG portion disulfide bonds have also been found to be more efficient in binding and neutralizing other molecules than monomeric polypeptides or fragments thereof alone. See, e.g., Fountoulakis et al., *J. Biochem.*, 270:3958-3964 (1995). Nucleic acids encoding the

above epitopes can also be recombined with a gene of interest as an epitope tag (e.g., the hemagglutinin ("HA") tag or flag tag) to aid in detection and purification of the expressed polypeptide. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972- 897). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six histidine residues. The tag serves as a matrix binding domain for the fusion protein. Extracts from cells infected with the recombinant vaccinia virus are loaded onto Ni<sup>2+</sup>-nitriloacetic acid-agarose column and histidine-tagged proteins can be selectively eluted with imidazole-containing buffers.

Additional fusion proteins of the invention may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling may be employed to modulate the activities of polypeptides of the invention, such methods can be used to generate polypeptides with altered activity, as well as agonists and antagonists of the polypeptides. See, generally, U.S. Patent Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Patten et al., Curr. Opin. Biotechnol. 8:724-33 (1997); Harayama, Trends Biotechnol. 16(2):76-82 (1998); Hansson, et al., J. Mol. Biol. 287:265-76 (1999); and Lorenzo and Blasco, Biotechniques 24(2):308- 13 (1998) (each of these patents and publications are hereby incorporated by reference in its entirety). In one embodiment, alteration of polynucleotides corresponding to SEQ ID NO:1 or 3 and the polypeptides encoded by these polynucleotides may be achieved by DNA shuffling. DNA shuffling involves the assembly of two or more DNA segments by homologous or site-specific recombination to generate variation in the polynucleotide sequence. In another embodiment, polynucleotides of the invention, or the encoded polypeptides, may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of a polynucleotide encoding a polypeptide of the invention may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

### **Antibodies**

Further polypeptides of the invention relate to antibodies and T-cell antigen receptors (TCR) which immunospecifically bind a polypeptide, polypeptide fragment, or variant of SEQ ID NOS:2 or 4, and/or an epitope, of the present invention (as determined

by immunoassays well known in the art for assaying specific antibody-antigen binding). Antibodies of the invention include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, anti-  
5 idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above. The term "antibody," as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that immunospecifically binds an antigen. The immunoglobulin molecules of the invention  
10 can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

Most preferably the antibodies are human antigen-binding antibody fragments of the present invention and include, but are not limited to, Fab, Fab' and F(ab')<sub>2</sub>, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and  
15 fragments comprising either a VL or VH domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, and  
20 CH3 domains. The antibodies of the invention may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine (e.g., mouse and rat), donkey, sheep rabbit, goat, guinea pig, camel, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries  
25 or from animals transgenic for one or more human immunoglobulin and that do not express endogenous immunoglobulins, as described infra and, for example in, U.S. Patent No. 5,939,598 by Kucherlapati et al.

The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for  
30 different epitopes of a polypeptide of the present invention or may be specific for both a polypeptide of the present invention as well as for a heterologous epitope, such as a heterologous polypeptide or solid support material. See, e.g., PCT publications WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, et al., J. Immunol. 147:60-69 (1991); U.S. Patent Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920;  
35 5,601,819; Kostelny et al., J. Immunol. 148:1547-1553 (1992).

Antibodies of the present invention may be described or specified in terms of the epitope(s) or portion(s) of a polypeptide of the present invention which they recognize or

specifically bind. The epitope(s) or polypeptide portion(s) may be specified as described herein, e.g., by N-terminal and C-terminal positions, by size in contiguous amino acid residues, or listed in the Tables and Figures.

In one embodiment, antigenic polypeptides or peptides that can be used to generate PDGF-A-specific antibodies include the following: a polypeptide comprising amino acid residues from about Lys-72 to about Leu-80 in SEQ ID NO:2, from about Ile-82 to about Ile-88 in SEQ ID NO:2, from about Pro-106 to about Ser-114 in SEQ ID NO:2, from about Lys-139 to about Val-145 in SEQ ID NO:2, from about Arg-159 to about Lys-165 in SEQ ID NO:2, and from about Ser-183 to about Thr-211 in SEQ ID NO:2. These polypeptide fragments have been determined to bear antigenic epitopes of the PDGF-A protein by the analysis of the Jameson-Wolf antigenic index using the default parameters.

In a preferred embodiment, the antigenic epitopes include the amino acid residues from about Ile-82 to about Ile-88 in SEQ ID NO:2.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate PDGF-B-specific antibodies include the following: a polypeptide comprising amino acid from about Arg-39 to about Gln-45 in SEQ ID NO:4, from about Asp-51 to about Asp-56 in SEQ ID NO:4, from about Thr-65 to about Gly-71 in SEQ ID NO:4, from about Leu-110 to about Ala-116 in SEQ ID NO:4, from about Ser-131 to about Thr-144 in SEQ ID NO:4, from about Pro-188 to about Thr-206 in SEQ ID NO:4, from about Arg-216 to about Lys-227 in SEQ ID NO:4, and from about Thr-229 to about Leu-235 in SEQ ID NO:4. These polypeptide fragments have been determined to bear antigenic epitopes of the PDGF-B protein by the analysis of the Jameson-Wolf antigenic index using the default parameters.

In a preferred embodiment, the antigenic epitopes include the amino acid residues from about Arg-39 to about Gln-45 in SEQ ID NO:4, from about Asp-51 to about Asp-56 in SEQ ID NO:4, and from about Thr-65 to about Gly-71 in SEQ ID NO:4.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate PIGF-specific antibodies include the following: a polypeptide comprising amino acid residues from about Val-46 to about Cys-52 in SEQ ID NO:6, from about Arg-110 to about Ser-116 in SEQ ID NO:6, and from about Val-126 to about Asp-144 in SEQ ID NO:6. These polypeptide fragments have been determined to bear antigenic epitopes of the PIGF protein by the analysis of the Jameson-Wolf antigenic index using the default parameters.

In a preferred embodiment, the antigenic epitopes include the amino acid residues from about Val-46 to about Cys-52 in SEQ ID NO:6.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate PlGF-2-specific antibodies include the following: a polypeptide comprising amino acid residues from about Val-46 to about Cys-52 in SEQ ID NO:8, from about Arg-110 to about Ser-116 in SEQ ID NO:8, and from about Val-126 to about Asp-159 in SEQ ID NO:8. These polypeptide fragments have been determined to bear antigenic epitopes of the PlGF-2 protein by the analysis of the Jameson-Wolf antigenic index using the default parameters.

In a preferred embodiment, the antigenic epitopes include the amino acid residues from about Val-46 to about Cys-52 in SEQ ID NO:8.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate GDGF-specific antibodies include the following: a polypeptide comprising amino acid residues from about Thr-30 to about Ala-36 in SEQ ID NO:10, from about Tyr-46 to about Cys-51 in SEQ ID NO:10, from about Glu-63 to about Ile-68 in SEQ ID NO:10, from about Ser-125 to about Cys-142 in SEQ ID NO:10, from about Pro-144 to about His-151 in SEQ ID NO:10, from about Gln-155 to about Lys-172 in SEQ ID NO:10, and from about Asn-179 to about Arg-190 in SEQ ID NO:10. These polypeptide fragments have been determined to bear antigenic epitopes of the GDGF protein by the analysis of the Jameson-Wolf antigenic index using the default parameters.

In a preferred embodiment, the antigenic epitopes include the amino acid residues from about Thr-30 to about Ala-36 in SEQ ID NO:10, and from about Tyr-46 to about Cys-51 in SEQ ID NO:10.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate VEGF-specific antibodies include the following: a polypeptide comprising amino acid residues from about Glu-64 to about Ile-69 in SEQ ID NO:12, from about Gly-85 to about Gly-91 in SEQ ID NO:12, from about His-125 to about Cys-143 in SEQ ID NO:12, from about Pro-145 to about His-152 in SEQ ID NO:12, from about Gln-156 to about Lys-173 in SEQ ID NO:12, and from about Asn-180 to about Arg-191 in SEQ ID NO:12. These polypeptide fragments have been determined to bear antigenic epitopes of the VEGF protein by the analysis of the Jameson-Wolf antigenic index using the default parameters.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate VEGF-A-specific antibodies include the following: a polypeptide comprising amino acid residues from about Thr-30 to about Ala-36 in SEQ ID NO:14, from about Tyr-46 to about Cys-51 in SEQ ID NO:14, from about Glu-63 to about Ile-68 in SEQ ID NO:14, from about Ser-125 to about Val-143 in SEQ ID NO:14, from about Gly-145 to about Cys-166 in SEQ ID NO:14, from about Pro-168 to about His-175 in SEQ ID NO:14, from about Gln-179 to about Lys-196 in SEQ ID NO:14, and from about Asn-

203 to about Arg-214 in SEQ ID NO:14. These polypeptide fragments have been determined to bear antigenic epitopes of the VEGF-A protein by the analysis of the Jameson-Wolf antigenic index using the default parameters.

In a preferred embodiment, the antigenic epitopes include the amino acid residues  
5 from about Thr-30 to about Ala-36 in SEQ ID NO:14, and from about Tyr-46 to about Cys-51 in SEQ ID NO:14.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate VEGF-2-specific antibodies include the following: a polypeptide comprising amino acid residues from about Asp-40 to about Ala-45 in SEQ ID NO:16, from about  
10 Lys-54 to about Leu-60 in SEQ ID NO:16, from about Gln-84 to about Gly-89 in SEQ ID NO:16, from about Arg-94 to about Thr-106 in SEQ ID NO:16, from about Ile-122 to about Cys-131 in SEQ ID NO:16, from about Asn-175 to about Leu-181 in SEQ ID NO:16, from about Gln-237 to about Pro-244 in SEQ ID NO:16, from about Asp-267 to about Phe-276 in SEQ ID NO:16, from about Pro-282 to about Asp-287 in SEQ ID  
15 NO:16, from about Ser-303 to about Ser-314 in SEQ ID NO:16, from about Ala-330 to about Thr-338 in SEQ ID NO:16, from about Arg-345 to about Cys-358 in SEQ ID NO:16, from about Gly-373 to about Cys-395 in SEQ ID NO:16, and from about Pro-410 to about Ser-419 in SEQ ID NO:16. These polypeptide fragments have been determined to bear antigenic epitopes of the VEGF-2 protein by the analysis of the  
20 Jameson-Wolf antigenic index using the default parameters.

In a preferred embodiment, the antigenic epitopes include the amino acid residues from about Asp-40 to about Ala-45 in SEQ ID NO:16, from about Lys-54 to about Leu-60 in SEQ ID NO:16, from about Gln-84 to about Gly-89 in SEQ ID NO:16, from about Arg-94 to about Thr-106 in SEQ ID NO:16, and from about Ile-122 to about Cys-131 in  
25 SEQ ID NO:16.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate VEGF-3-specific antibodies include the following: a polypeptide comprising amino acid residues from about Ser-24 to about Lys-34 in SEQ ID NO:18, from about Ala-45 to about Arg-50 in SEQ ID NO:18, from about Arg-125 to about Ala-138 in SEQ  
30 ID NO:18, from about Pro-141 to about Ser-149 in SEQ ID NO:18, and from about His-167 to about Pro-172 in SEQ ID NO:18. These polypeptide fragments have been determined to bear antigenic epitopes of the VEGF-3 protein by the analysis of the Jameson-Wolf antigenic index using the default parameters.

In a preferred embodiment, the antigenic epitopes include the amino acid residues  
35 from about Ser-24 to about Lys-34 in SEQ ID NO:18, and from about Ala-45 to about Arg-50 in SEQ ID NO:18.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate VEGF-D-specific antibodies include the following: a polypeptide comprising amino acid residues from about Met-1 to about Gln-14 in SEQ ID NO:20, from about His-29 to about Arg-41 in SEQ ID NO:20, from about Met-48 to about Thr-58 in SEQ ID NO:20, from about Glu-75 to about Thr-87 in SEQ ID NO:20, from about Leu-95 to about Phe-103 in SEQ ID NO:20, from about Pro-181 to about Lys-191 in SEQ ID NO:20, from about Trp-199 to about Cys-204 in SEQ ID NO:20, from about Asp-281 to about Thr-293 in SEQ ID NO:20, from about Pro-310 to about Ala-316 in SEQ ID NO:20, and from about Gly-318 to about Pro-325 in SEQ ID NO:20. These polypeptide fragments have been determined to bear antigenic epitopes of the VEGF-D protein by the analysis of the Jameson-Wolf antigenic index using the default parameters.

In a preferred embodiment, the antigenic epitopes include the amino acid residues from about Met-1 to about Gln-14 in SEQ ID NO:20, from about His-29 to about Arg-41 in SEQ ID NO:20, from about Met-48 to about Thr-58 in SEQ ID NO:20, and from about Glu-75 to about Thr-87 in SEQ ID NO:20.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate VEGF-D1-specific antibodies include the following: a polypeptide comprising amino acid residues from about Ser-22 to about Gln-43 in SEQ ID NO:22, from about His-58 to about Arg-70 in SEQ ID NO:22, from about Met-77 to about Thr-87 in SEQ ID NO:22, from about Glu-104 to about Thr-116 in SEQ ID NO:22, from about Leu-124 to about Phe-132 in SEQ ID NO:22, from about Pro-210 to about Lys-220 in SEQ ID NO:22, from about Trp-228 to about Cys-233 in SEQ ID NO:22, from about Asp-310 to about Thr-322 in SEQ ID NO:22, from about Pro-339 to about Ala-345 in SEQ ID NO:22, and from about Gly-347 to about Pro-354 in SEQ ID NO:22. These polypeptide fragments have been determined to bear antigenic epitopes of the VEGF-D protein by the analysis of the Jameson-Wolf antigenic index using the default parameters.

In a preferred embodiment, the antigenic epitopes include the amino acid residues from about Ser-22 to about Gln-43 in SEQ ID NO:22, from about His-58 to about Arg-70 in SEQ ID NO:22, and from about Met-77 to about Thr-87 in SEQ ID NO:22.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate VEGF-E-specific antibodies include the following: a polypeptide comprising amino acid residues from about Asp-21 to about Trp-26 in SEQ ID NO:24, from about Asp-46 to about Phe-56 in SEQ ID NO:24, from about Gly-68 to about Ser-74 in SEQ ID NO:24, and from about Pro-121 to about Arg-132 in SEQ ID NO:24. These polypeptide fragments have been determined to bear antigenic epitopes of the VEGF-E protein by the analysis of the Jameson-Wolf antigenic index using the default parameters.

In a preferred embodiment, the antigenic epitopes include the amino acid residues from about Asp-21 to about Trp-26 in SEQ ID NO:24.

Antibodies which specifically bind any epitope or polypeptide of the present invention may also be excluded. Therefore, the present invention includes antibodies that specifically bind polypeptides of the present invention, and allows for the exclusion of the same.

Antibodies of the present invention may also be described or specified in terms of their cross-reactivity. Antibodies that do not bind any other analog, ortholog, or homolog of a polypeptide of the present invention are included. Antibodies that bind polypeptides with at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, and at least 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention. In specific embodiments, antibodies of the present invention cross-react with murine, rat and/or rabbit homologs of human proteins and the corresponding epitopes thereof. Antibodies that do not bind polypeptides with less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, and less than 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention. In a specific embodiment, the above-described cross-reactivity is with respect to any single specific antigenic or immunogenic polypeptide, or combination(s) of 2, 3, 4, 5, or more of the specific antigenic and/or immunogenic polypeptides disclosed herein. Further included in the present invention are antibodies which bind polypeptides encoded by polynucleotides which hybridize to a polynucleotide of the present invention under stringent hybridization conditions (as described herein). Antibodies of the present invention may also be described or specified in terms of their binding affinity to a polypeptide of the invention. Preferred binding affinities include those with a dissociation constant or  $K_d$  less than  $5 \times 10^{-2}$  M,  $10^{-2}$  M,  $5 \times 10^{-3}$  M,  $10^{-3}$  M,  $5 \times 10^{-4}$  M,  $10^{-4}$  M,  $5 \times 10^{-5}$  M,  $10^{-5}$  M,  $5 \times 10^{-6}$  M,  $10^{-6}$  M,  $5 \times 10^{-7}$  M,  $10^{-7}$  M,  $5 \times 10^{-8}$  M,  $10^{-8}$  M,  $5 \times 10^{-9}$  M,  $10^{-9}$  M,  $5 \times 10^{-10}$  M,  $10^{-10}$  M,  $5 \times 10^{-11}$  M,  $10^{-11}$  M,  $5 \times 10^{-12}$  M,  $10^{-12}$  M,  $5 \times 10^{-13}$  M,  $10^{-13}$  M,  $5 \times 10^{-14}$  M,  $10^{-14}$  M,  $5 \times 10^{-15}$  M, or  $10^{-15}$  M.

The invention also provides antibodies that competitively inhibit binding of an antibody to an epitope of the invention as determined by any method known in the art for determining competitive binding, for example, the immunoassays described herein. In preferred embodiments, the antibody competitively inhibits binding to the epitope by at least 95%, at least 90%, at least 85 %, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50%.



Antibodies of the present invention may act as agonists or antagonists of the polypeptides of the present invention. For example, the present invention includes antibodies which disrupt the receptor/ligand interactions with the polypeptides of the invention either partially or fully. Preferably, antibodies of the present invention bind an antigenic epitope disclosed herein, or a portion thereof. The invention features both  
5 receptor-specific antibodies and ligand-specific antibodies. The invention also features receptor-specific antibodies which do not prevent ligand binding but prevent receptor activation. Receptor activation (i.e., signaling) may be determined by techniques described herein or otherwise known in the art. For example, receptor activation can be  
10 determined by detecting the phosphorylation (e.g., tyrosine or serine/threonine) of the receptor or its substrate by immunoprecipitation followed by western blot analysis (for example, as described *supra*). In specific embodiments, antibodies are provided that inhibit ligand activity or receptor activity by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50% of the activity in  
15 absence of the antibody.

The invention also features receptor-specific antibodies which both prevent ligand binding and receptor activation as well as antibodies that recognize the receptor-ligand complex, and, preferably, do not specifically recognize the unbound receptor or the unbound ligand. Likewise, included in the invention are neutralizing antibodies which  
20 bind the ligand and prevent binding of the ligand to the receptor, as well as antibodies which bind the ligand, thereby preventing receptor activation, but do not prevent the ligand from binding the receptor. Further included in the invention are antibodies which activate the receptor. These antibodies may act as receptor agonists, i.e., potentiate or activate either all or a subset of the biological activities of the ligand-mediated receptor  
25 activation, for example, by inducing dimerization of the receptor. The antibodies may be specified as agonists, antagonists or inverse agonists for biological activities comprising the specific biological activities of the peptides of the invention disclosed herein. The above antibody agonists can be made using methods known in the art. See, e.g., PCT publication WO 96/40281; U.S. Patent No. 5,811,097; Deng et al., Blood 92(6):1981-  
30 1988 (1998); Chen et al., Cancer Res. 58(16):3668-3678 (1998); Harrop et al., J. Immunol. 161(4):1786-1794 (1998); Zhu et al., Cancer Res. 58(15):3209-3214 (1998); Yoon et al., J. Immunol. 160(7):3170-3179 (1998); Prat et al., J. Cell. Sci. 111(Pt2):237-247 (1998); Pitard et al., J. Immunol. Methods 205(2):177-190 (1997); Liautard et al., Cytokine 9(4):233-241 (1997); Carlson et al., J. Biol. Chem.  
35 272(17):11295-11301 (1997); Taryman et al., Neuron 14(4):755-762 (1995); Muller et al., Structure 6(9):1153-1167 (1998); Bartunek et al., Cytokine 8(1):14-20 (1996) (which are all incorporated by reference herein in their entireties).

Antibodies of the present invention may be used, for example, but not limited to, to purify, detect, and target the polypeptides of the present invention, including both in vitro and in vivo diagnostic and therapeutic methods. For example, the antibodies have use in immunoassays for qualitatively and quantitatively measuring levels of the polypeptides of the present invention in biological samples. See, e.g., Harlow et al.,  
5 Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988) (incorporated by reference herein in its entirety).

As discussed in more detail below, the antibodies of the present invention may be used either alone or in combination with other compositions. The antibodies may further  
10 be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or chemically conjugated (including covalently and non-covalently conjugations) to polypeptides or other compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as labels in detection assays and effector molecules such as heterologous polypeptides, drugs, radionuclides,  
15 or toxins. See, e.g., PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Patent No. 5,314,995; and EP 396,387.

The antibodies of the invention include derivatives that are modified, i.e., by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from generating an anti-idiotypic response. For example,  
20 but not by way of limitation, the antibody derivatives include antibodies that have been modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage,  
25 acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

The antibodies of the present invention may be generated by any suitable method known in the art. Polyclonal antibodies to an antigen-of-interest can be produced by various procedures well known in the art. For example, a polypeptide of the invention  
30 can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances  
35 such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG

(bacille Calmette-Guerin) and *corynebacterium parvum*. Such adjuvants are also well known in the art.

Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art and are discussed in detail in the Examples. In a non-limiting example, mice can be immunized with a polypeptide of the invention or a cell expressing such peptide. Once an immune response is detected, e.g., antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding a polypeptide of the invention. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.

Accordingly, the present invention provides methods of generating monoclonal antibodies as well as antibodies produced by the method comprising culturing a hybridoma cell secreting an antibody of the invention wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from a mouse immunized with an antigen of the invention with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind a polypeptide of the invention.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and F(ab')<sub>2</sub> fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')<sub>2</sub> fragments). F(ab')<sub>2</sub>

fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

For example, the antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al., *J. Immunol. Methods* 182:41-50 (1995); Ames et al., *J. Immunol. Methods* 184:177-186 (1995); Kettleborough et al., *Eur. J. Immunol.* 24:952-958 (1994); Persic et al., *Gene* 187 9-18 (1997); Burton et al., *Advances in Immunology* 57:191-280 (1994); PCT application No. PCT/GB91/01134; PCT publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')<sub>2</sub> fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax et al., *BioTechniques* 12(6):864-869 (1992); and Sawai et al., *AJRI* 34:26-34 (1995); and Better et al., *Science* 240:1041-1043 (1988) (said references incorporated by reference in their entireties).

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston et al., *Methods in Enzymology* 203:46-88 (1991); Shu et al., *PNAS* 90:7995-7999 (1993); and Skerra et al., *Science* 240:1038-1040 (1988). For some uses, including in vivo use

of antibodies in humans and in vitro detection assays, it may be preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. See e.g., Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Gillies et al., (1989) J. Immunol. Methods 125:191-202; U.S. Patent Nos. 5,807,715; 4,816,567; and 4,816,397, which are incorporated herein by reference in their entirety. Humanized antibodies are antibody molecules from non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and a framework regions from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Patent No. 5,585,089; Riechmann et al., Nature 332:323 (1988), which are incorporated herein by reference in their entirety.) Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089); veneering or resurfacing (EP 592,106; EP 519,596; Padlan, Molecular Immunology 28(4/5):489-498 (1991); Studnicka et al., Protein Engineering 7(6):805-814 (1994); Roguska, et al., PNAS 91:969-973 (1994)), and chain shuffling (U.S. Patent No. 5,565,332).

Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety.

Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable

region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar, *Int. Rev. Immunol.* 13:65-93 (1995). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers et al., *Bio/technology* 12:899-903 (1988)).

Further, antibodies to the polypeptides of the invention can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" polypeptides of the invention using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, *FASEB J.* 7(5):437-444; (1989) and Nissinoff, J. *Immunol.* 147(8):2429-2438 (1991)). For example, antibodies which bind to and competitively inhibit polypeptide multimerization and/or binding of a polypeptide of the invention to a ligand can be used to generate anti-idiotypes that "mimic" the polypeptide multimerization and/or binding domain and, as a

consequence, bind to and neutralize polypeptide and/or its ligand. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize polypeptide ligand. For example, such anti-idiotypic antibodies can be used to bind a polypeptide of the invention and/or to bind its ligands/receptors, and thereby  
5 block its biological activity.

### **Polynucleotides Encoding Antibodies**

The invention further provides polynucleotides comprising a nucleotide sequence encoding an antibody of the invention and fragments thereof. The invention also  
10 encompasses polynucleotides that hybridize under stringent or lower stringency hybridization conditions, e.g., as defined *supra*, to polynucleotides that encode an antibody, preferably, that specifically binds to a polypeptide of the invention, preferably, an antibody that binds to a polypeptide having the amino acid sequence of SEQ ID NOS:2 or 4.

15 The polynucleotides may be obtained, and the nucleotide sequence of the polynucleotides determined, by any method known in the art. For example, if the nucleotide sequence of the antibody is known, a polynucleotide encoding the antibody may be assembled from chemically synthesized oligonucleotides (e.g., as described in Kutmeier et al., BioTechniques 17:242 (1994)), which, briefly, involves the synthesis of  
20 overlapping oligonucleotides containing portions of the sequence encoding the antibody, annealing and ligating of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, a polynucleotide encoding an antibody may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid encoding a  
25 particular antibody is not available, but the sequence of the antibody molecule is known, a nucleic acid encoding the immunoglobulin may be chemically synthesized or obtained from a suitable source (e.g., an antibody cDNA library, or a cDNA library generated from, or nucleic acid, preferably poly A+ RNA, isolated from, any tissue or cells expressing the antibody, such as hybridoma cells selected to express an antibody of the  
30 invention) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence to identify, e.g., a cDNA clone from a cDNA library that encodes the antibody. Amplified nucleic acids generated by PCR may then be cloned into replicable cloning vectors using any method well known in the art.

35 Once the nucleotide sequence and corresponding amino acid sequence of the antibody is determined, the nucleotide sequence of the antibody may be manipulated using methods well known in the art for the manipulation of nucleotide sequences, e.g.,

recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, for example, the techniques described in Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY and Ausubel et al., eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY, which are both incorporated by reference herein in their entireties ), to generate antibodies having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or insertions.

In a specific embodiment, the amino acid sequence of the heavy and/or light chain variable domains may be inspected to identify the sequences of the complementarity determining regions (CDRs) by methods that are well known in the art, e.g., by comparison to known amino acid sequences of other heavy and light chain variable regions to determine the regions of sequence hypervariability. Using routine recombinant DNA techniques, one or more of the CDRs may be inserted within framework regions, e.g., into human framework regions to humanize a non-human antibody, as described *supra*. The framework regions may be naturally occurring or consensus framework regions, and preferably human framework regions (see, e.g., Chothia et al., J. Mol. Biol. 278: 457-479 (1998) for a listing of human framework regions). Preferably, the polynucleotide generated by the combination of the framework regions and CDRs encodes an antibody that specifically binds a polypeptide of the invention. Preferably, as discussed *supra*, one or more amino acid substitutions may be made within the framework regions, and, preferably, the amino acid substitutions improve binding of the antibody to its antigen. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more variable region cysteine residues participating in an intrachain disulfide bond to generate antibody molecules lacking one or more intrachain disulfide bonds. Other alterations to the polynucleotide are encompassed by the present invention and within the skill of the art.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., Proc. Natl. Acad. Sci. 81:851-855 (1984); Neuberger et al., Nature 312:604-608 (1984); Takeda et al., Nature 314:452-454 (1985)) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. As described *supra*, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region, e.g., humanized antibodies.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778; Bird, Science 242:423-42 (1988); Huston et al., Proc. Natl. Acad. Sci. USA 85:5879-5883 (1988); and Ward et al., Nature 334:544-54 (1989))



can be adapted to produce single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in *E. coli* may also be used (Skerra et al., Science 242:1038- 1041 (1988)).

5

### **Methods of Producing Antibodies**

The antibodies of the invention can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or preferably, by recombinant expression techniques.

10

Recombinant expression of an antibody of the invention, or fragment, derivative or analog thereof, (e.g., a heavy or light chain of an antibody of the invention or a single chain antibody of the invention), requires construction of an expression vector containing a polynucleotide that encodes the antibody. Once a polynucleotide encoding an antibody molecule or a heavy or light chain of an antibody, or portion thereof (preferably containing the heavy or light chain variable domain), of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an antibody encoding nucleotide sequence are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding an antibody molecule of the invention, or a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (see, e.g., PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464) and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy or light chain.

30

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody of the invention. Thus, the invention includes host cells containing a polynucleotide encoding an antibody of the invention, or a heavy or light chain thereof, or a single chain antibody of the invention, operably linked to a heterologous promoter. In preferred embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

35

A variety of host-expression vector systems may be utilized to express the antibody molecules of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express an antibody molecule of the invention in situ. These include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing antibody coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter). Preferably, bacterial cells such as *Escherichia coli*, and more preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecule, are used for the expression of a recombinant antibody molecule. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies (Foecking et al., *Gene* 45:101 (1986); Cockett et al., *Bio/Technology* 8:2 (1990)).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., *EMBO J.* 2:1791 (1983)), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, *Nucleic Acids Res.* 13:3101-3109 (1985); Van Heeke & Schuster, *J. Biol. Chem.* 24:5503-5509 (1989)); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and

can easily be purified from lysed cells by adsorption and binding to matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

5 In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

10 In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination.  
15 Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody molecule in infected hosts. (e.g., see Logan & Shenk, Proc. Natl. Acad. Sci. USA 81:355-359 (1984)). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and  
20 adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc.  
25 (see Bittner et al., Methods in Enzymol. 153:51-544 (1987)).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g.,  
30 cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and  
35 phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, breast cancer cell lines such as, for example, BT483, Hs578T, HTB2, BT20

and T47D, and normal mammary gland cell line such as, for example, CRL7030 and Hs578Bst.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the antibody molecule may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the antibody molecule. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibody molecule.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., Cell 11:223 (1977)), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, Proc. Natl. Acad. Sci. USA 48:202 (1992)), and adenine phosphoribosyltransferase (Lowy et al., Cell 22:817 (1980)) genes can be employed in tk-, hgp<sup>r</sup>t- or ap<sup>r</sup>t- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., Natl. Acad. Sci. USA 77:357 (1980); O'Hare et al., Proc. Natl. Acad. Sci. USA 78:1527 (1981)); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, Proc. Natl. Acad. Sci. USA 78:2072 (1981)); neo, which confers resistance to the aminoglycoside G-418 Clinical Pharmacy 12:488-505; Wu and Wu, Biotherapy 3:87-95 (1991); Tolstoshev, Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993); Mulligan, Science 260:926-932 (1993); and Morgan and Anderson, Ann. Rev. Biochem. 62:191-217 (1993); May, 1993, TIB TECH 11(5):155-215; and hyg<sup>r</sup>, which confers resistance to hygromycin (Santerre et al., Gene 30:147 (1984)). Methods commonly known in the art of recombinant DNA technology may be routinely applied to select the desired recombinant clone, and such methods are described, for example, in Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990); and in Chapters 12 and 13, Dracopoli et al. (eds), Current Protocols in Human Genetics, John Wiley & Sons,

NY (1994); Colberre-Garapin et al., *J. Mol. Biol.* 150:1 (1981), which are incorporated by reference herein in their entireties.

The expression levels of an antibody molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, *The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning*, Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody will also increase (Crouse et al., *Mol. Cell. Biol.* 3:257 (1983)).

The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes, and is capable of expressing, both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, *Nature* 322:52 (1986); Kohler, *Proc. Natl. Acad. Sci. USA* 77:2197 (1980)). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once an antibody molecule of the invention has been produced by an animal, chemically synthesized, or recombinantly expressed, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In addition, the antibodies of the present invention or fragments thereof can be fused to heterologous polypeptide sequences described herein or otherwise known in the art, to facilitate purification.

The present invention encompasses antibodies recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations) to a polypeptide (or portion thereof, preferably at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids of the polypeptide) of the present invention to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences. The antibodies may be specific for antigens other than polypeptides (or portion thereof, preferably at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids of the polypeptide) of the present invention. For example, antibodies may be used to target the polypeptides of the present invention to particular cell types, either in vitro or in vivo, by

fusing or conjugating the polypeptides of the present invention to antibodies specific for particular cell surface receptors. Antibodies fused or conjugated to the polypeptides of the present invention may also be used in in vitro immunoassays and purification methods using methods known in the art. See e.g., Harbor et al., *supra*, and PCT publication WO 93/21232; EP 439,095; Naramura et al., *Immunol. Lett.* 39:91-99 (1994); U.S. Patent 5,474,981; Gillies et al., *PNAS* 89:1428-1432 (1992); Fell et al., *J. Immunol.* 146:2446-2452(1991), which are incorporated by reference in their entireties.

The present invention further includes compositions comprising the polypeptides of the present invention fused or conjugated to antibody domains other than the variable regions. For example, the polypeptides of the present invention may be fused or conjugated to an antibody Fc region, or portion thereof. The antibody portion fused to a polypeptide of the present invention may comprise the constant region, hinge region, CH1 domain, CH2 domain, and CH3 domain or any combination of whole domains or portions thereof. The polypeptides may also be fused or conjugated to the above antibody portions to form multimers. For example, Fc portions fused to the polypeptides of the present invention can form dimers through disulfide bonding between the Fc portions. Higher multimeric forms can be made by fusing the polypeptides to portions of IgA and IgM. Methods for fusing or conjugating the polypeptides of the present invention to antibody portions are known in the art. See, e.g., U.S. Patent Nos. 5,336,603; 5,622,929; 5,359,046; 5,349,053; 5,447,851; 5,112,946; EP 307,434; EP 367,166; PCT publications WO 96/04388; WO 91/06570; Ashkenazi et al., *Proc. Natl. Acad. Sci. USA* 88:10535-10539 (1991); Zheng et al., *J. Immunol.* 154:5590-5600 (1995); and Vil et al., *Proc. Natl. Acad. Sci. USA* 89:11337-11341(1992) (said references incorporated by reference in their entireties).

As discussed, *supra*, the polypeptides corresponding to a polypeptide, polypeptide fragment, or a variant of SEQ ID NOS:2 or 4 may be fused or conjugated to the above antibody portions to increase the in vivo half life of the polypeptides or for use in immunoassays using methods known in the art. Further, the polypeptides corresponding to SEQ ID NOS:2 or 4 may be fused or conjugated to the above antibody portions to facilitate purification. One reported example describes chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. (EP 394,827; Traunecker et al., *Nature* 331:84-86 (1988)). The polypeptides of the present invention fused or conjugated to an antibody having disulfide-linked dimeric structures (due to the IgG) may also be more efficient in binding and neutralizing other molecules, than the monomeric secreted protein or protein fragment alone. (Fountoulakis et al., *J. Biochem.* 270:3958-3964 (1995)). In many cases, the Fc part in a fusion protein is

beneficial in therapy and diagnosis, and thus can result in, for example, improved pharmacokinetic properties. (EP A 232,262). Alternatively, deleting the Fc part after the fusion protein has been expressed, detected, and purified, would be desired. For example, the Fc portion may hinder therapy and diagnosis if the fusion protein is used as an antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. (See, Bennett et al., J. Molecular Recognition 8:52-58 (1995); Johanson et al., J. Biol. Chem. 270:9459-9471 (1995)).

Moreover, the antibodies or fragments thereof of the present invention can be fused to marker sequences, such as a peptide to facilitate purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz et al., Proc. Natl. Acad. Sci. USA 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., Cell 37:767 (1984)) and the "flag" tag.

The present invention further encompasses antibodies or fragments thereof conjugated to a diagnostic or therapeutic agent. The antibodies can be used diagnostically to, for example, monitor the development or progression of a tumor as part of a clinical testing procedure to, e.g., determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions. The detectable substance may be coupled or conjugated either directly to the antibody (or fragment thereof) or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art. See, for example, U.S. Patent No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics according to the present invention. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include

luciferase, luciferin, and aequorin; and examples of suitable radioactive material include <sup>125</sup>I, <sup>131</sup>I, <sup>111</sup>In or <sup>99</sup>Tc.

Further, an antibody or fragment thereof may be conjugated to a therapeutic moiety such as a cytotoxin, e.g., a cytostatic or cytotoxic agent, a therapeutic agent or a radioactive metal ion, e.g., alpha-emitters such as, for example, <sup>213</sup>Bi. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological response, the therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, alpha-interferon, beta-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, an apoptotic agent, e.g., TNF-alpha, TNF-beta, AIM I (See, International Publication No. WO 97/33899), AIM II (See, International Publication No. WO 97/34911), Fas Ligand (Takahashi *et al.*, *Int. Immunol.*, 6:1567-1574 (1994)), VEGF (See, International Publication No. WO 99/23105), a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Antibodies may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but



are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in *Controlled Drug Delivery (2nd Ed.)*, Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.* 62:119-58 (1982).

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980, which is incorporated herein by reference in its entirety.

An antibody, with or without a therapeutic moiety conjugated to it, administered alone or in combination with cytotoxic factor(s) and/or cytokine(s) can be used as a therapeutic.

### **Immunophenotyping**

The antibodies of the invention may be utilized for immunophenotyping of cell lines and biological samples. The translation product of the gene of the present invention may be useful as a cell specific marker, or more specifically as a cellular marker that is differentially expressed at various stages of differentiation and/or maturation of particular cell types. Monoclonal antibodies directed against a specific epitope, or combination of epitopes, will allow for the screening of cellular populations expressing the marker. Various techniques can be utilized using monoclonal antibodies to screen for cellular populations expressing the marker(s), and include magnetic separation using antibody-coated magnetic beads, "panning" with antibody attached to a solid matrix (i.e., plate), and flow cytometry (See, e.g., U.S. Patent 5,985,660; and Morrison *et al.*, *Cell*, 96:737-49 (1999)).

These techniques allow for the screening of particular populations of cells, such as might be found with hematological malignancies (i.e. minimal residual disease (MRD) in acute leukemic patients) and "non-self" cells in transplantations to prevent Graft-versus-Host Disease (GVHD). Alternatively, these techniques allow for the screening of

hematopoietic stem and progenitor cells capable of undergoing proliferation and/or differentiation, as might be found in human umbilical cord blood.

### **Assays For Antibody Binding**

5       The antibodies of the invention may be assayed for immunospecific binding by any method known in the art. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel  
10       diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein  
15       in its entirety). Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

      Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X- 100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1%  
20       Trasylol) supplemented with protein phosphatase and/or protease inhibitors (e.g., EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody of interest to the cell lysate, incubating for a period of time (e.g., 1-4 hours) at 4° C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 4° C, washing the beads in lysis buffer and resuspending the beads in SDS/sample buffer.  
25       The ability of the antibody of interest to immunoprecipitate a particular antigen can be assessed by, e.g., western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to an antigen and decrease the background (e.g., pre-clearing the cell lysate with sepharose beads). For further discussion regarding immunoprecipitation protocols see, e.g.,  
30       Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.16.1.

      Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (e.g., 8%- 20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample  
35       from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution (e.g., PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (e.g., PBS-Tween 20), blocking the membrane

with primary antibody (the antibody of interest) diluted in blocking buffer, washing the membrane in washing buffer, blocking the membrane with a secondary antibody (which recognizes the primary antibody, e.g., an anti-human antibody) conjugated to an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) or radioactive molecule (e.g.,  $^{32}\text{P}$  or  $^{125}\text{I}$ ) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the antigen. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For further discussion regarding western blot protocols see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.8.1.

ELISAs comprise preparing antigen, coating the well of a 96 well microtiter plate with the antigen, adding the antibody of interest conjugated to a detectable compound such as an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. ELISAs the antibody of interest does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added following the addition of the antigen of interest to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1.

The binding affinity of an antibody to an antigen and the off-rate of an antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (e.g.,  $^3\text{H}$  or  $^{125}\text{I}$ ) with the antibody of interest in the presence of increasing amounts of unlabeled antigen, and the detection of the antibody bound to the labeled antigen. The affinity of the antibody of interest for a particular antigen and the binding off-rates can be determined from the data by scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, the antigen is incubated with antibody of interest conjugated to a labeled compound (e.g.,  $^3\text{H}$  or  $^{125}\text{I}$ ) in the presence of increasing amounts of an unlabeled second antibody.

### Therapeutic Uses

The present invention is further directed to antibody-based therapies which involve administering antibodies of the invention to an animal, preferably a mammal, and most preferably a human, patient for treating one or more of the disclosed diseases, disorders, or conditions. Therapeutic compounds of the invention include, but are not limited to, antibodies of the invention (including fragments, analogs and derivatives thereof as described herein) and nucleic acids encoding antibodies of the invention (including fragments, analogs and derivatives thereof and anti-idiotypic antibodies as described herein). The antibodies of the invention can be used to treat, inhibit or prevent diseases, disorders or conditions associated with aberrant expression and/or activity of a polypeptide of the invention, including, but not limited to, any one or more of the diseases, disorders, or conditions described herein. The treatment and/or prevention of diseases, disorders, or conditions associated with aberrant expression and/or activity of a polypeptide of the invention includes, but is not limited to, alleviating symptoms associated with those diseases, disorders or conditions. Antibodies of the invention may be provided in pharmaceutically acceptable compositions as known in the art or as described herein.

A summary of the ways in which the antibodies of the present invention may be used therapeutically includes binding polynucleotides or polypeptides of the present invention locally or systemically in the body or by direct cytotoxicity of the antibody, e.g. as mediated by complement (CDC) or by effector cells (ADCC). Some of these approaches are described in more detail below. Armed with the teachings provided herein, one of ordinary skill in the art will know how to use the antibodies of the present invention for diagnostic, monitoring or therapeutic purposes without undue experimentation.

The antibodies of this invention may be advantageously utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines or hematopoietic growth factors (such as, e.g., IL-2, IL-3 and IL-7), for example, which serve to increase the number or activity of effector cells which interact with the antibodies.

The antibodies of the invention may be administered alone or in combination with other types of treatments (e.g., radiation therapy, chemotherapy, hormonal therapy, immunotherapy and anti-tumor agents). Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, human antibodies, fragments derivatives, analogs, or nucleic acids, are administered to a human patient for therapy or prophylaxis.

It is preferred to use high affinity and/or potent in vivo inhibiting and/or neutralizing antibodies against polypeptides or polynucleotides of the present invention, fragments or regions thereof, for both immunoassays directed to and therapy of disorders related to polynucleotides or polypeptides, including fragments thereof, of the present invention. Such antibodies, fragments, or regions, will preferably have an affinity for polynucleotides or polypeptides of the invention, including fragments thereof. Preferred binding affinities include those with a dissociation constant or  $K_d$  less than  $5 \times 10^{-2}$  M,  $10^{-2}$  M,  $5 \times 10^{-3}$  M,  $10^{-3}$  M,  $5 \times 10^{-4}$  M,  $10^{-4}$  M,  $5 \times 10^{-5}$  M,  $10^{-5}$  M,  $5 \times 10^{-6}$  M,  $10^{-6}$  M,  $5 \times 10^{-7}$  M,  $10^{-7}$  M,  $5 \times 10^{-8}$  M,  $10^{-8}$  M,  $5 \times 10^{-9}$  M,  $10^{-9}$  M,  $5 \times 10^{-10}$  M,  $10^{-10}$  M,  $5 \times 10^{-11}$  M,  $10^{-11}$  M,  $5 \times 10^{-12}$  M,  $10^{-12}$  M,  $5 \times 10^{-13}$  M,  $10^{-13}$  M,  $5 \times 10^{-14}$  M,  $10^{-14}$  M,  $5 \times 10^{-15}$  M, and  $10^{-15}$  M.

### Gene Therapy

In a specific embodiment, nucleic acids comprising sequences encoding antibodies or functional derivatives thereof, are administered to treat, inhibit or prevent a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acids produce their encoded protein that mediates a therapeutic effect.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., Clinical Pharmacy 12:488-505 (1993); Wu and Wu, Biotherapy 3:87-95 (1991); Tolstoshev, Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993); Mulligan, Science 260:926-932 (1993); and Morgan and Anderson, Ann. Rev. Biochem. 62:191-217 (1993); May, TIBTECH 11(5):155-215 (1993). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); and Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

In a preferred aspect, the compound comprises nucleic acid sequences encoding an antibody, said nucleic acid sequences being part of expression vectors that express the antibody or fragments or chimeric proteins or heavy or light chains thereof in a suitable host. In particular, such nucleic acid sequences have promoters operably linked to the antibody coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, nucleic acid molecules are used in which the antibody coding sequences and any other desired sequences are flanked by

regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the antibody encoding nucleic acids (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); Zijlstra et al., Nature 342:435-438 (1989). In specific embodiments, the expressed antibody molecule is a single chain antibody; alternatively, the nucleic acid sequences include sequences encoding both the heavy and light chains, or fragments thereof, of the antibody.

Delivery of the nucleic acids into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids in vitro, then transplanted into the patient. These two approaches are known, respectively, as in vivo or ex vivo gene therapy.

In a specific embodiment, the nucleic acid sequences are directly administered in vivo, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, e.g., by constructing them as part of an appropriate nucleic acid expression vector and administering it so that they become intracellular, e.g., by infection using defective or attenuated retrovirals or other viral vectors (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering them in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, e.g., Wu and Wu, J. Biol. Chem. 262:4429-4432 (1987)) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted in vivo for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180; WO 92/22635; WO92/20316; WO93/14188, WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); Zijlstra et al., Nature 342:435-438 (1989)).

In a specific embodiment, viral vectors that contains nucleic acid sequences encoding an antibody of the invention are used. For example, a retroviral vector can be used (see Miller et al., Meth. Enzymol. 217:581-599 (1993)). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding the antibody to

be used in gene therapy are cloned into one or more vectors, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., *Biotherapy* 6:291-302 (1994), which describes the use of a retroviral vector to deliver the *mdr1* gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., *J. Clin. Invest.* 93:644-651 (1994); Kiem et al., *Blood* 83:1467-1473 (1994); Salmons and Gunzberg, *Human Gene Therapy* 4:129-141 (1993); and Grossman and Wilson, *Curr. Opin. in Genetics and Devel.* 3:110-114 (1993).

Adenoviruses are other viral vectors that can be used in gene therapy.

Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, *Current Opinion in Genetics and Development* 3:499-503 (1993) present a review of adenovirus-based gene therapy. Bout et al., *Human Gene Therapy* 5:3-10 (1994) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., *Science* 252:431-434 (1991); Rosenfeld et al., *Cell* 68:143-155 (1992); Mastrangeli et al., *J. Clin. Invest.* 91:225-234 (1993); PCT Publication WO94/12649; and Wang, et al., *Gene Therapy* 2:775-783 (1995). In a preferred embodiment, adenovirus vectors are used.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., *Proc. Soc. Exp. Biol. Med.* 204:289-300 (1993); U.S. Patent No. 5,436,146).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration in vivo of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the

art for the introduction of foreign genes into cells (see, e.g., Loeffler and Behr, Meth. Enzymol. 217:599-618 (1993); Cohen et al., Meth. Enzymol. 217:618-644 (1993); Cline, Pharmac. Ther. 29:69-92m (1985) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. Recombinant blood cells (e.g., hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as Tlymphocytes, Blymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, e.g., as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy, nucleic acid sequences encoding an antibody are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered in vivo for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained in vitro can potentially be used in accordance with this embodiment of the present invention (see e.g. PCT Publication WO 94/08598; Stemple and Anderson, Cell 71:973-985 (1992); Rheinwald, Meth. Cell Bio. 21A:229 (1980); and Pittelkow and Scott, Mayo Clinic Proc. 61:771 (1986)).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription. Demonstration of Therapeutic or Prophylactic Activity

The compounds or pharmaceutical compositions of the invention are preferably tested in vitro, and then in vivo for the desired therapeutic or prophylactic activity, prior



to use in humans. For example, in vitro assays to demonstrate the therapeutic or prophylactic utility of a compound or pharmaceutical composition include, the effect of a compound on a cell line or a patient tissue sample. The effect of the compound or composition on the cell line and/or tissue sample can be determined utilizing techniques known to those of skill in the art including, but not limited to, rosette formation assays and cell lysis assays. In accordance with the invention, in vitro assays which can be used to determine whether administration of a specific compound is indicated, include in vitro cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a compound, and the effect of such compound upon the tissue sample is observed.

#### **Therapeutic/Prophylactic Administration and Composition**

The invention provides methods of treatment, inhibition and prophylaxis by administration to a subject of an effective amount of a compound or pharmaceutical composition of the invention, preferably an antibody of the invention. In a preferred aspect, the compound is substantially purified (e.g., substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid or an immunoglobulin are described above; additional appropriate formulations and routes of administration can be selected from among those described herein below.

Various delivery systems are known and can be used to administer a compound of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, e.g., Wu and Wu, J. Biol. Chem. 262:4429-4432 (1987)), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds or compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compounds or compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular

catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compounds or compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when administering a protein, including an antibody, of the invention, care must be taken to use materials to which the protein does not absorb.

In another embodiment, the compound or composition can be delivered in a vesicle, in particular a liposome (see Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*)

In yet another embodiment, the compound or composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Press, Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J., *Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); see also Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al., *J. Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered in vivo to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by use of a retroviral

vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot et al., Proc. Natl. Acad. Sci. USA 88:1864-1868 (1991)), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration

are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the compound of the invention which will be effective in the treatment, inhibition and prevention of a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention can be determined by standard clinical techniques. In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

For antibodies, the dosage administered to a patient is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. Preferably, the dosage administered to a patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight, more preferably 1 mg/kg to 10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the invention may be reduced by enhancing uptake and tissue penetration (e.g., into the brain) of the antibodies by modifications such as, for example, lipidation.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions

of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

5

### **Diagnosis and Imaging**

Labeled antibodies, and derivatives and analogs thereof, which specifically bind to a polypeptide of interest can be used for diagnostic purposes to detect, diagnose, or monitor diseases, disorders, and/or conditions associated with the aberrant expression and/or activity of a polypeptide of the invention. The invention provides for the detection of aberrant expression of a polypeptide of interest, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of aberrant expression.

The invention provides a diagnostic assay for diagnosing a disorder, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of a particular disorder. With respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

Antibodies of the invention can be used to assay protein levels in a biological sample using classical immunohistological methods known to those of skill in the art (e.g., see Jalkanen, et al., J. Cell. Biol. 101:976-985 (1985); Jalkanen, et al., J. Cell Biol. 105:3087-3096 (1987)). Other antibody-based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase; radioisotopes, such as iodine (125I, 121I), carbon (14C), sulfur (35S), tritium (3H),

indium ( $^{112}\text{In}$ ), and technetium ( $^{99}\text{Tc}$ ); luminescent labels, such as luminol; and fluorescent labels, such as fluorescein and rhodamine, and biotin.

One aspect of the invention is the detection and diagnosis of a disease or disorder associated with aberrant expression of a polypeptide of interest in an animal, preferably a mammal and most preferably a human. In one embodiment, diagnosis comprises: a) administering (for example, parenterally, subcutaneously, or intraperitoneally) to a subject an effective amount of a labeled molecule which specifically binds to the polypeptide of interest; b) waiting for a time interval following the administering for permitting the labeled molecule to preferentially concentrate at sites in the subject where the polypeptide is expressed (and for unbound labeled molecule to be cleared to background level); c) determining background level; and d) detecting the labeled molecule in the subject, such that detection of labeled molecule above the background level indicates that the subject has a particular disease or disorder associated with aberrant expression of the polypeptide of interest. Background level can be determined by various methods including, comparing the amount of labeled molecule detected to a standard value previously determined for a particular system.

It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of  $^{99\text{m}}\text{Tc}$ . The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain the specific protein. In vivo tumor imaging is described in S.W. Burchiel et al., "Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments." (Chapter 13 in *Tumor Imaging: The Radiochemical Detection of Cancer*, S.W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982).

Depending on several variables, including the type of label used and the mode of administration, the time interval following the administration for permitting the labeled molecule to preferentially concentrate at sites in the subject and for unbound labeled molecule to be cleared to background level is 6 to 48 hours or 6 to 24 hours or 6 to 12 hours. In another embodiment the time interval following administration is 5 to 20 days or 5 to 10 days.

In an embodiment, monitoring of the disease or disorder is carried out by repeating the method for diagnosing the disease or disorder, for example, one month after initial diagnosis, six months after initial diagnosis, one year after initial diagnosis, etc.

Presence of the labeled molecule can be detected in the patient using methods known in the art for in vivo scanning. These methods depend upon the type of label used. Skilled artisans will be able to determine the appropriate method for detecting a

particular label. Methods and devices that may be used in the diagnostic methods of the invention include, but are not limited to, computed tomography (CT), whole body scan such as position emission tomography (PET), magnetic resonance imaging (MRI), and sonography.

5 In a specific embodiment, the molecule is labeled with a radioisotope and is detected in the patient using a radiation responsive surgical instrument (Thurston et al., U.S. Patent No. 5,441,050). In another embodiment, the molecule is labeled with a fluorescent compound and is detected in the patient using a fluorescence responsive scanning instrument. In another embodiment, the molecule is labeled with a positron  
10 emitting metal and is detected in the patient using positron emission-tomography. In yet another embodiment, the molecule is labeled with a paramagnetic label and is detected in a patient using magnetic resonance imaging (MRI).

### Kits

15 The present invention provides kits that can be used in the above methods. In one embodiment, a kit comprises an antibody of the invention, preferably a purified antibody, in one or more containers. In a specific embodiment, the kits of the present invention contain a substantially isolated polypeptide comprising an epitope which is specifically immunoreactive with an antibody included in the kit. Preferably, the kits of the present  
20 invention further comprise a control antibody which does not react with the polypeptide of interest. In another specific embodiment, the kits of the present invention contain a means for detecting the binding of an antibody to a polypeptide of interest (e.g., the antibody may be conjugated to a detectable substrate such as a fluorescent compound, an enzymatic substrate, a radioactive compound or a luminescent compound, or a second  
25 antibody which recognizes the first antibody may be conjugated to a detectable substrate).

In another specific embodiment of the present invention, the kit is a diagnostic kit for use in screening serum containing antibodies specific against proliferative and/or cancerous polynucleotides and polypeptides. Such a kit may include a control antibody that does not react with the polypeptide of interest. Such a kit may include a substantially  
30 isolated polypeptide antigen comprising an epitope which is specifically immunoreactive with at least one anti-polypeptide antigen antibody. Further, such a kit includes means for detecting the binding of said antibody to the antigen (e.g., the antibody may be conjugated to a fluorescent compound such as fluorescein or rhodamine which can be detected by flow cytometry). In specific embodiments, the kit may include a  
35 recombinantly produced or chemically synthesized polypeptide antigen. The polypeptide antigen of the kit may also be attached to a solid support.

In a more specific embodiment the detecting means of the above-described kit includes a solid support to which said polypeptide antigen is attached. Such a kit may also include a non-attached reporter-labeled anti-human antibody. In this embodiment, binding of the antibody to the polypeptide antigen can be detected by binding of the said reporter-labeled antibody.

In an additional embodiment, the invention includes a diagnostic kit for use in screening serum containing antigens of the polypeptide of the invention. The diagnostic kit includes a substantially isolated antibody specifically immunoreactive with polypeptide or polynucleotide antigens, and means for detecting the binding of the polynucleotide or polypeptide antigen to the antibody. In one embodiment, the antibody is attached to a solid support. In a specific embodiment, the antibody may be a monoclonal antibody. The detecting means of the kit may include a second, labeled monoclonal antibody. Alternatively, or in addition, the detecting means may include a labeled, competing antigen.

In one diagnostic configuration, test serum is reacted with a solid phase reagent having a surface-bound antigen obtained by the methods of the present invention. After binding with specific antigen antibody to the reagent and removing unbound serum components by washing, the reagent is reacted with reporter-labeled anti-human antibody to bind reporter to the reagent in proportion to the amount of bound anti-antigen antibody on the solid support. The reagent is again washed to remove unbound labeled antibody, and the amount of reporter associated with the reagent is determined. Typically, the reporter is an enzyme which is detected by incubating the solid phase in the presence of a suitable fluorometric, luminescent or colorimetric substrate (Sigma, St. Louis, MO).

The solid surface reagent in the above assay is prepared by known techniques for attaching protein material to solid support material, such as polymeric beads, dip sticks, 96-well plate or filter material. These attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein, typically through a free amine group, to a chemically reactive group on the solid support, such as an activated carboxyl, hydroxyl, or aldehyde group. Alternatively, streptavidin coated plates can be used in conjunction with biotinylated antigen(s).

Thus, the invention provides an assay system or kit for carrying out this diagnostic method. The kit generally includes a support with surface-bound recombinant antigens, and a reporter-labeled anti-human antibody for detecting surface-bound anti-antigen antibody.



***Polynucleotides***

Isolated nucleic acid molecules comprising the polynucleotides of the present invention include: Platelet Derived Growth Factor-A (PDGF-A), as disclosed in European Patent Number EP-682110 (SEQ ID NO: 1); Platelet Derived Growth Factor-B (PDGF-B), as disclosed in European Patent Number EP-282317 (SEQ ID NO: 3);  
5 Placental Growth Factor (PIGF), as disclosed in International Publication Number WO 92/06194 (SEQ ID NO: 5); Placental Growth Factor-2 (PIGF-2), as disclosed in Häuser *et al.*, *Growth Factors*, 4:259-268 (1993) (SEQ ID NO: 7); Glioma Derived Growth Factor (GDGF), as disclosed in European Patent Number EP-399816 (SEQ ID NO: 9);  
10 Vascular Endothelial Growth Factor (VEGF), as disclosed in International Publication Number WO 90/13649 (SEQ ID NO: 11); Vascular Endothelial Growth Factor-A (VEGF-A), as disclosed in European Patent Number EP-506477 (SEQ ID NO: 13); Vascular Endothelial Growth Factor-2 (VEGF-2), as disclosed in International Publication Number WO 96/39515 (SEQ ID NO: 15); Vascular Endothelial Growth  
15 Factor-3 (VEGF-3), as disclosed in International Publication Number WO 96/39421 (SEQ ID NO: 17); Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/07832 (SEQ ID NO: 19); Vascular Endothelial Growth Factor-D1 (VEGF-D1), as disclosed in International Publication Number WO 98/02543 (SEQ ID NO: 21); and Vascular Endothelial Growth Factor-E (VEGF-E), as  
20 disclosed in German Patent Number DE19639601 (SEQ ID NO: 23). The above mentioned references are herein incorporated by reference in their entirety.

The angiogenic proteins of the present invention are all structurally related. It is particularly important that all eight cysteines are conserved within all members of the VEGF/PDGF families (see boxed areas of Figures 3A-3C). In addition, the signature for  
25 the PDGF/VEGF families, PXCXXXXRCXGCC, (SEQ ID NO:29) is conserved in all angiogenic proteins disclosed herein (see Figures 3A-3C).

The angiogenic polypeptides of the present invention include the full length polypeptides and polynucleotide sequences which encode for any leader sequence and for active fragments of the full length polypeptides.

30 The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence of the nucleotide sequences  
35 of Table 1, or may be a different coding sequence which, as a result of the redundancy or degeneracy of the genetic code, encodes the same, mature polypeptide as the DNA of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23.

The polynucleotides which encode for the respective mature polypeptides of Figures 3A-3C may include: only the coding sequences for the respective mature polypeptides; the coding sequences for the respective mature polypeptides and additional coding sequences such as a leader or secretory sequence or a proprotein sequence; the coding sequences for the respective mature polypeptides (and optionally additional coding sequences) and non-coding sequences, such as introns or non-coding sequence 5' and/or 3' of the coding sequences for the mature polypeptides.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequences for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequences.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs, and derivatives of the polypeptide having the deduced amino acid sequence of Figures 3A-3C. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotides or a non-naturally occurring variant of the polynucleotides.

Thus, the present invention includes polynucleotides encoding the same respective mature polypeptides as shown in Figures 3A-3C as well as variants of such polynucleotides which variants encode for fragments, derivatives, or analogs of the polypeptides of Figures 3A-3C. Such nucleotide variants include deletion variants, substitution variants, and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and presequence (leader sequence).

5 The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, *e.g.* COS-7 cells, is used. The HA tag corresponds to  
10 an epitope derived from the influenza hemagglutinin protein (Wilson, I., *et al.*, *Cell* 37:767 (1984)).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence  
15 encoding the polypeptide having the amino acid sequence in SEQ ID NO:2; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 211 in SEQ ID NO:2; or (d) a nucleotide sequence complementary to any of the nucleotide  
20 sequences in (a), (b), or (c).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:4; (b) a  
25 nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:4, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 241 in SEQ ID NO:4; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

30 Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:6; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID  
35 NO:6, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 149 in

SEQ ID NO:6; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:8; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:8, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 170 in SEQ ID NO:8; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:10; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:10, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 190 in SEQ ID NO:10; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:12; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:12, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 191 in SEQ ID NO:12; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:14; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:14, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 214 in

SEQ ID NO:14; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:16; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:16, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 419 in SEQ ID NO:16; (d) a nucleotide sequence encoding the polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No.97149; (e) a nucleotide sequence encoding the mature VEGF2 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No.97149; (f) a nucleotide sequence encoding the polypeptide encoded by the cDNA clone contained in ATCC Deposit No.97149, but lacking the N-terminal Methionine; or (g) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), or (f).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:18; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:18, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 207 in SEQ ID NO:18; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:20; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:20, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 325 in SEQ ID NO:20; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and

more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:22; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:22, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 354 in SEQ ID NO:22; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:24; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:24, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 132 in SEQ ID NO:24; or (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c).

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding an angiogenic polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the angiogenic polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23 can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2: 482-489 (1981), to find the best segment of homology between two sequences. When

using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in  
5 homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

An angiogenic "polynucleotide" also includes those polynucleotides capable of hybridizing, under stringent hybridization conditions, to sequences contained in SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23, or for instance, the cDNA clone(s)  
10 contained in ATCC Deposit Nos. 97149 or 75698, or the complement thereof.

"Stringent hybridization conditions" refers to an overnight incubation at 42° C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x  
15 SSC at about 65°C.

Nucleic acid molecules may also hybridize to the angiogenic polynucleotides at lower stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency); salt  
20 conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2M NaH<sub>2</sub>PO<sub>4</sub>; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking DNA; followed by washes at 50°C with 1XSSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent  
25 hybridization can be done at higher salt concentrations (e.g. 5X SSC).

Note that variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available  
30 proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

Of course, a polynucleotide which hybridizes only to polyA+ sequences (such as any 3' terminal polyA+ tract of a cDNA shown in the sequence listing), or to a  
35 complementary stretch of T (or U) residues, would not be included in the definition of "polynucleotide," since such a polynucleotide would hybridize to any nucleic acid

molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15  
5 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

By a portion of a polynucleotide of "at least 20 nt in length," for example, is  
10 intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the nucleotide sequence of Table 1). Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3' terminal poly(A) tract of SEQ ID NO:X), or to a complementary stretch of T (or U) residues, would not be included in a  
15 polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

Preferred, however, are nucleic acid molecules having sequences at least 95%,  
96%, 97%, 98% or 99% identical to a nucleic acid sequence shown in SEQ ID NOS: 1,  
20 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23, or to a nucleic acid sequence of the deposited cDNA(s) which do, in fact, encode a polypeptide having angiogenic protein activity. By "a polypeptide having angiogenic activity" is intended polypeptides exhibiting angiogenic activity in a particular biological assay. For example, angiogenic protein activity can be measured using, for example, mitogenic assays and endothelial cell migration assays.  
25 See, e.g., Olofsson *et al.*, *Proc. Natl. Acad. Sci. USA* 93:2576-2581 (1996) and Joukov *et al.*, *EMBO J.* 5:290-298 (1996). By "a polypeptide having lymphangiogenic activity" is intended polypeptides exhibiting lymphangiogenic activity in a particular biological assay. For example, angiogenic protein activity can be measured using, for example, mitogenic assays and endothelial cell migration assays. See, e.g., Olofsson *et al.*, *Proc.*  
30 *Natl. Acad. Sci. USA* 93:2576-2581 (1996) and Joukov *et al.*, *EMBO J.* 5:290-298 (1996).

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to a nucleic acid  
35 sequence of the deposited cDNA(s) or the nucleic acid sequences shown in SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23 will encode a polypeptide "having angiogenic protein activity." In fact, since degenerate variants of these nucleotide sequences all



encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having angiogenic protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U. *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95%, 96%, 97%, or 98% identity to a polynucleotide which encodes the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24, as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

"Identity" per se has an art-recognized meaning and can be calculated using published techniques. (See, e.g.: (COMPUTATIONAL MOLECULAR BIOLOGY, Lesk, A.M., ed., Oxford University Press, New York, (1988); BIOCOMPUTING: INFORMATICS AND GENOME PROJECTS, Smith, D.W., ed., Academic Press, New York, (1993); COMPUTER ANALYSIS OF SEQUENCE DATA, PART I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, (1994); SEQUENCE ANALYSIS IN MOLECULAR BIOLOGY, von Heinje, G., Academic Press, (1987); and SEQUENCE ANALYSIS PRIMER, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, (1991).) While there exists a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans. (Carillo, H., and Lipton, D., *SIAM J. Applied Math.* 48:1073 (1988).) Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in "Guide to Huge Computers," Martin J. Bishop, ed., Academic Press, San Diego, (1994), and Carillo, H., and Lipton, D., *SIAM J. Applied Math.* 48:1073 (1988). Methods for aligning polynucleotides or polypeptides are codified in computer programs, including the GCG program package (Devereux, J., *et al.*, *Nucleic Acids Research* 12(1):387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S.F. *et al.*, *J. Molec. Biol.* 215:403 (1990), Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for

Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711 (using the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2:482-489 (1981)). By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence of the present invention, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the angiogenic polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. The query sequence may be an entire sequence of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23, the ORF (open reading frame), or any fragment specified as described herein.

As a practical matter, whether any particular nucleic acid molecule or polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleotide sequence of the present invention can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag *et al.* (*Comp. App. Biosci.* 6:237-245 (1990)). In a sequence alignment the query and subject sequences are both DNA sequences. An RNA sequence can be compared by converting U's to T's. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. Whether a nucleotide is matched/aligned is determined by results of the

FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of the present invention. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score.

For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 bases at 5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to made for the purposes of the present invention.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, (indels) or substituted with another amino acid. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequences shown in Table 1 or to the amino acid sequence encoded by deposited DNA clone can be determined conventionally using known computer programs. A preferred method for

determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag *et al.* (*Comp. App. Biosci.* (1990) 6:237-245). In a sequence alignment the query and subject sequences are either both nucleotide sequences or both amino acid sequences. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. Whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present invention. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence.

For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C-termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent

identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to be made for the purposes of the present invention.

### ***Angiogenic Polypeptides***

The present invention further relates to polypeptides which have the deduced amino acid sequence of Figures 3A-3C, as well as fragments, analogs, and derivatives of such polypeptides.

In the present invention, a "polypeptide fragment" refers to a short amino acid sequence contained in SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24, or encoded by the cDNA contained in a deposited clone. Protein fragments may be "free-standing," or comprised within a larger polypeptide of which the fragment forms a part or region, most preferably as a single continuous region. Representative examples of polypeptide fragments of the invention, include, for example, fragments from about amino acid number 1-20, 21-40, 41-60, 61-80, 81-100, 102-120, 121-140, 141-160, 161-180, 181-200, 201-220, 221-240, 241-260, 261-280, or 281 to the end of the coding region. Moreover, polypeptide fragments can be about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 amino acids in length. In this context "about" includes the particularly recited ranges, larger or smaller by several (5, 4, 3, 2, or 1) amino acids, at either extreme or at both extremes (See Table 1).

TABLE 1

Gene No.	Gene Name	NT SEQ ID NO:X	Total NT Seq.	5' NT of Start Codon	AA SEQ ID NO:Y	Last AA of ORF
1	PDGF-A	1	1308	388	2	211
2	PDGF-B	3	2137	983	4	241
3	PIGF	5	1645	322	6	149
4	PIGF-2	7	597	13	8	170
5	GDGF	9	577	5	10	190
6	VEGF	11	989	56	12	191
7	VEGF-A	13	649	5	14	214
8	VEGF-2	15	1674	12	16	419
9	VEGF-3	17	624	1	18	207
10	VEGF-D	19	2846	1771	20	325
11	VEGF-D1	21	2004	403	22	354
12	VEGF-E	23	399	1	24	132

Preferred polypeptide fragments include the secreted angiogenic protein as well as the mature form. Further preferred polypeptide fragments include the secreted angiogenic protein or the mature form having a continuous series of deleted residues from the amino or the carboxy terminus, or both. For example, any number of amino acids, ranging from 1-60, can be deleted from the amino terminus of either the secreted angiogenic polypeptide or the mature form. Similarly, any number of amino acids, ranging from 1-30, can be deleted from the carboxy terminus of the secreted angiogenic protein or mature form. Furthermore, any combination of the above amino and carboxy terminus deletions are preferred. Similarly, polynucleotide fragments encoding these angiogenic polypeptide fragments are also preferred.

Also preferred are angiogenic polypeptide and polynucleotide fragments characterized by structural or functional domains, such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn

and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions. Polypeptide fragments of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24 falling within conserved domains are specifically contemplated by the present invention. (See Figures 3A-3C). Moreover, polynucleotide fragments encoding these domains are also contemplated.

Other preferred fragments are biologically active angiogenic protein fragments. Biologically active fragments are those exhibiting activity similar, but not necessarily identical, to an activity of one of the disclosed angiogenic polypeptides. The biological activity of the fragments may include an improved desired activity, or a decreased undesirable activity.

The polypeptides of the present invention may be recombinant polypeptides, natural polypeptides, or synthetic polypeptides, preferably recombinant polypeptides.

It will be recognized in the art that some amino acid sequences of the angiogenic polypeptides can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity.

Thus, the invention further includes variations of the angiogenic polypeptides which show substantial angiogenic polypeptide activity or which include regions of angiogenic proteins such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U., *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990).

Thus, the fragments, derivatives, or analogs of the polypeptides of Table 1 may be: (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code; or (ii) one in which one or more of the amino acid residues includes a substituent group; or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol); or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence; or (v) one in which comprises fewer amino acid residues shown in SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24, and retains the conserved motif and yet still retains activity

characteristics of the VEGF/PDGF family of polypeptides. Such fragments, derivatives, and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Of particular interest are substitutions of charged amino acids with another charged amino acid and with neutral or negatively charged amino acids. The latter results in proteins with reduced positive charge to improve the characteristics of the angiogenic protein. The prevention of aggregation is highly desirable. Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical formulations, because they can be immunogenic. (Pinckard *et al.*, *Clin. Exp. Immunol.* 2:331-340 (1967); Robbins *et al.*, *Diabetes* 36:838-845 (1987); Cleland *et al. Crit. Rev. Therapeutic Drug Carrier Systems* 10:307-377 (1993)).

The replacement of amino acids can also change the selectivity of binding to cell surface receptors. Ostade *et al.*, *Nature* 361:266-268 (1993) describes certain mutations resulting in selective binding of TNF- $\alpha$  to only one of the two known types of TNF receptors. Thus, the angiogenic proteins of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 2).

**TABLE 2. Conservative Amino Acid Substitutions**

Aromatic	Phenylalanine, Tryptophan, Tyrosine
Hydrophobic	Leucine, Isoleucine, Valine
Polar	Glutamine, Asparagine
Basic	Arginine, Lysine, Histidine
Acidic	Aspartic Acid, Glutamic Acid
Small	Alanine, Serine, Threonine, Methionine, Glycine

Of course, the number of amino acid substitutions a skilled artisan would make depends on many factors, including those described above. Generally speaking, the number of substitutions for any given angiogenic polypeptide will not be more than 50, 40, 30, 25, 20, 15, 10, 5 or 3.

Amino acids in the angiogenic proteins of the present invention that are essential for function can be identified by methods known in the art, such as site-directed



mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or *in vitro*, or *in vitro* proliferative activity.

5 Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al.*, *J. Mol. Biol.* 224:899-904 (1992) and de Vos *et al.* *Science* 255:306-312 (1992)).

10 The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or DNA or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotide could be part of a vector and/or such polynucleotide or polypeptide could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

20 In specific embodiments, the polynucleotides of the invention are less than 300 kb, 200 kb, 100 kb, 50 kb, 15 kb, 10 kb, or 7.5 kb in length. In a further embodiment, polynucleotides of the invention comprise at least 15 contiguous nucleotides of the coding sequence of a disclosed angiogenic protein, but do not comprise all or a portion of any intron of the particular angiogenic protein. In another embodiment, the nucleic acid comprising a particular angiogenic protein coding sequence does not contain coding sequences of a genomic flanking gene (*i.e.*, 5' or 3' to the particular angiogenic protein gene in the genome).

30 The polypeptides of the present invention include the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24 (and in particular the mature polypeptide thereof), as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24, and more preferably at least 90% similarity (more preferably at least 95% identity) to the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24, and still more preferably at least 95% similarity (still more preferably at least 90% identity) to the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

#### 10 *Amino terminal and carboxy terminal deletions*

In addition, protein engineering may be employed in order to improve or alter one or more characteristics of native angiogenic proteins. The deletion of carboxy terminal amino acids can enhance the activity of proteins. One example is interferon gamma that shows up to ten times higher activity by deleting ten amino acid residues from the carboxy terminus of the protein (Döbeli *et al.*, *J. of Biotechnology* 7:199-216 (1988)). Thus, one aspect of the invention is to provide polypeptide analogs of angiogenic proteins and nucleotide sequences encoding such analogs that exhibit enhanced stability (e.g., when exposed to typical pH, thermal conditions or other storage conditions) relative to the native angiogenic protein.

Also included by the present invention are deletion mutants having amino acids deleted from both the N-terminus and the C-terminus. Such mutants include all combinations of N-terminal deletion mutants and C-terminal deletion mutants described by the formulas below. Those combinations can be made using recombinant techniques known to those skilled in the art.

Particularly, N-terminal deletions of angiogenic polypeptides can be described by the general formulas below:

1.)  $m_2$ -211, where  $m_2$  is an integer from 1 to 210, where  $m_2$  corresponds to the position of the amino acid residue identified in SEQ ID NO:2. Moreover, C-terminal deletions can also be described by the general formula 1- $n_2$ , where  $n_2$  is an integer from 2 to 211 where  $n_2$  corresponds to the position of amino acid residue identified in SEQ ID NO:2. Moreover, the invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_2$ - $n_2$  of SEQ ID NO:2, where  $n_2$  and  $m_2$  are integers as described above.

2.)  $m_4$ -241, where  $m_4$  is an integer from 1 to 240, where  $m_4$  corresponds to the position of the amino acid residue identified in SEQ ID NO:4. Moreover, C-terminal deletions can also be described by the general formula 1- $n_4$ , where  $n_4$  is an integer from 2

to 241 where  $n_4$  corresponds to the position of amino acid residue identified in SEQ ID NO:4. Moreover, the invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_4-n_4$  of SEQ ID NO:4, where  $n_4$  and  $m_4$  are integers as described above.

3.)  $m_6$ -149, where  $m_6$  is an integer from 1 to 148, where  $m_6$  corresponds to the position of the amino acid residue identified in SEQ ID NO:6. Moreover, C-terminal deletions can also be described by the general formula  $1-n_6$ , where  $n_6$  is an integer from 2 to 149 where  $n_6$  corresponds to the position of amino acid residue identified in SEQ ID NO:6. Moreover, the invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_6-n_6$  of SEQ ID NO:6, where  $n_6$  and  $m_6$  are integers as described above.

4.)  $m_8$ -170, where  $m_8$  is an integer from 1 to 169, where  $m_8$  corresponds to the position of the amino acid residue identified in SEQ ID NO:8. Moreover, C-terminal deletions can also be described by the general formula  $1-n_8$ , where  $n_8$  is an integer from 2 to 170 where  $n_8$  corresponds to the position of amino acid residue identified in SEQ ID NO:8. Moreover, the invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_8-n_8$  of SEQ ID NO:8, where  $n_8$  and  $m_8$  are integers as described above.

5.)  $m_{10}$ -190, where  $m_{10}$  is an integer from 1 to 189, where  $m_{10}$  corresponds to the position of the amino acid residue identified in SEQ ID NO:10. Moreover, C-terminal deletions can also be described by the general formula  $1-n_{10}$ , where  $n_{10}$  is an integer from 2 to 190 where  $n_{10}$  corresponds to the position of amino acid residue identified in SEQ ID NO:10. Moreover, the invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_{10}-n_{10}$  of SEQ ID NO:10, where  $n_{10}$  and  $m_{10}$  are integers as described above.

6.)  $m_{12}$ -191, where  $m_{12}$  is an integer from 1 to 190, where  $m_{12}$  corresponds to the position of the amino acid residue identified in SEQ ID NO:12. Moreover, C-terminal deletions can also be described by the general formula  $1-n_{12}$ , where  $n_{12}$  is an integer from 2 to 191 where  $n_{12}$  corresponds to the position of amino acid residue identified in SEQ ID NO:12. Moreover, the invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_{12}-n_{12}$  of SEQ ID NO:12, where  $n_{12}$  and  $m_{12}$  are integers as described above.

7.)  $m_{14}$ -214, where  $m_{14}$  is an integer from 1 to 213, where  $m_{14}$  corresponds to the position of the amino acid residue identified in SEQ ID NO:14. Moreover, C-terminal deletions can also be described by the general formula  $1-n_{14}$ , where  $n_{14}$  is an integer from 2 to 214 where  $n_{14}$  corresponds to the position of amino acid residue identified in SEQ ID NO:14. Moreover, the invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_{14}$ - $n_{14}$  of SEQ ID NO:14, where  $n_{14}$  and  $m_{14}$  are integers as described above.

8.)  $m_{16}$ -419, where  $m_{16}$  is an integer from 1 to 418, where  $m_{16}$  corresponds to the position of the amino acid residue identified in SEQ ID NO:16. Moreover, C-terminal deletions can also be described by the general formula  $1-n_{16}$ , where  $n_{16}$  is an integer from 2 to 419 where  $n_{16}$  corresponds to the position of amino acid residue identified in SEQ ID NO:16. Moreover, the invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_{16}$ - $n_{16}$  of SEQ ID NO:16, where  $n_{16}$  and  $m_{16}$  are integers as described above.

Specifically preferred is a polypeptide fragment comprising amino acid residues F-32 to R-226 of SEQ ID NO:16, as well as polynucleotides encoding this polypeptide. Also, another preferred embodiment comprises amino acids S-228 to S-419 of SEQ ID NO:16. Also preferred are polynucleotides encoding these polypeptides. Moreover, this F-32 to R-226 of SEQ ID NO:16 polypeptide preferably is associated with a S-228 to S-419 of SEQ ID NO:16 polypeptide. Association may be through disulfide, covalent, or noncovalent interactions, by linkage via a linker (e.g., serine, glycine, proline linkages), or by an antibody.

9.)  $m_{18}$ -207, where  $m_{18}$  is an integer from 1 to 206, where  $m_{18}$  corresponds to the position of the amino acid residue identified in SEQ ID NO:18. Moreover, C-terminal deletions can also be described by the general formula  $1-n_{18}$ , where  $n_{18}$  is an integer from 2 to 207 where  $n_{18}$  corresponds to the position of amino acid residue identified in SEQ ID NO:18. Moreover, the invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_{18}$ - $n_{18}$  of SEQ ID NO:18, where  $n_{18}$  and  $m_{18}$  are integers as described above.

10.)  $m_{20}$ -325, where  $m_{20}$  is an integer from 1 to 324, where  $m_{20}$  corresponds to the position of the amino acid residue identified in SEQ ID NO:20. Moreover, C-terminal deletions can also be described by the general formula  $1-n_{20}$ , where  $n_{20}$  is an integer from 2 to 325 where  $n_{20}$  corresponds to the position of amino acid residue identified in SEQ ID NO:20. Moreover, the invention also provides polypeptides having one or more amino

acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_{20}$ - $n_{20}$  of SEQ ID NO:20, where  $n_{20}$  and  $m_{20}$  are integers as described above.

11.)  $m_{22}$ -354, where  $m_{22}$  is an integer from 1 to 353, where  $m_{22}$  corresponds to the position of the amino acid residue identified in SEQ ID NO:22. Moreover, C-terminal deletions can also be described by the general formula 1- $n_{22}$ , where  $n_{22}$  is an integer from 2 to 354 where  $n_{22}$  corresponds to the position of amino acid residue identified in SEQ ID NO:22. Moreover, the invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_{22}$ - $n_{22}$  of SEQ ID NO:22, where  $n_{22}$  and  $m_{22}$  are integers as described above.

12.)  $m_{24}$ -132, where  $m_{24}$  is an integer from 1 to 131, where  $m_{24}$  corresponds to the position of the amino acid residue identified in SEQ ID NO:24. Moreover, C-terminal deletions can also be described by the general formula 1- $n_{24}$ , where  $n_{24}$  is an integer from 2 to 132 where  $n_{24}$  corresponds to the position of amino acid residue identified in SEQ ID NO:24. Moreover, the invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues  $m_{24}$ - $n_{24}$  of SEQ ID NO:24, where  $n_{24}$  and  $m_{24}$  are integers as described above.

In yet another aspect, also included by the present invention are deletion mutants having amino acids deleted from both the N-terminal and C-terminal residues. Such mutants include all combinations of the N-terminal deletion mutants and C-terminal deletion mutants described above.

In another aspect of the invention, mutants of the disclosed angiogenic polypeptide sequences are useful as screening agents to detect agonists and/or antagonists of angiogenic activity, or they can be modified to be used as an agonistic and/or antagonistic agent for the angiogenic activity of the disclosed angiogenic polypeptides, or can be used in a co-treatment application in conjunction with another agent, such as a monoclonal antibody specific for a disclosed angiogenic polypeptide, for example, the result of which may either be agonistic and/or antagonistic for the angiogenic activity of the disclosed angiogenic polypeptides.

In another aspect of the invention, fragments of the other referenced and disclosed angiogenic polypeptides of the invention, SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24, can be generated to produce mutants which may be useful as agonistic and/or antagonistic agents to modulate the activities of the disclosed angiogenic polypeptides. Furthermore, these polypeptide mutants may be used in a co-treatment

application with another agent, such as a monoclonal antibody specific for a disclosed angiogenic polypeptide, for example, to enhance or reduce a specific desired activity.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated nucleic acid molecule having the nucleotide sequence shown in SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23, is intended fragments at least about 15 nt, and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably, at least about 40 nt in length which are useful as diagnostic probes and primers as discussed herein. Of course, larger fragments of 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1025, 1050, 1075, 1100, 1125, 1150, 1175, 1200, 1225, 1250, 1275, 1300, 1325, 1350, 1375, 1400, 1425, 1450, 1475, 1500, 1525, 1550, 1575, 1600, 1625, or 1650 nt in length are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequences shown in SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23. By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from the nucleotide sequence shown in SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23. In this context "about" includes the particularly recited ranges, larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini. Preferably, these fragments encode a polypeptide which has biological activity.

Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA library to isolate the full length cDNA and to isolate other cDNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promoter regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

### ***Fusion Proteins***

Any disclosed angiogenic polypeptide can be used to generate fusion proteins. For example, angiogenic polypeptides, when fused to a second protein, can be used as an antigenic tag. Antibodies raised against the angiogenic polypeptide can be used to indirectly detect the second protein by binding to the angiogenic polypeptide. Moreover, because secreted proteins target cellular locations based on trafficking signals, the angiogenic polypeptides can be used as a targeting molecule once fused to other proteins.

Examples of domains that can be fused to disclosed angiogenic polypeptides include not only heterologous signal sequences, but also other heterologous functional regions. The fusion does not necessarily need to be direct, but may occur through linker sequences.

Moreover, fusion proteins may also be engineered to improve characteristics of one or more of the disclosed angiogenic polypeptides. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the angiogenic polypeptides to improve stability and persistence during purification from the host cell or subsequent handling and storage. Also, peptide moieties may be added to any of the disclosed angiogenic polypeptides to facilitate purification. Such regions may be removed prior to final preparation of the particular angiogenic polypeptide. The addition of peptide moieties to facilitate handling of polypeptides are familiar and routine techniques in the art.

Moreover, disclosed angiogenic polypeptides, including fragments, and specifically epitopes, can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life in vivo. One reported example describes chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. (EP A 394,827; Traunecker *et al.*, *Nature* 331:84-86 (1988).) Fusion proteins having disulfide-linked dimeric structures (due to the IgG) can also be more efficient in binding and neutralizing other molecules, than the monomeric secreted protein or protein fragment alone. (Fountoulakis *et al.*, *J. Biochem.* 270:3958-3964 (1995).)

Similarly, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is beneficial in therapy and diagnosis, and thus can result in, for example, improved pharmacokinetic properties. (EP-A 0232 262.) Alternatively, deleting the Fc part after the fusion protein has been expressed, detected, and purified, would be desired.

For example, the Fc portion may hinder therapy and diagnosis if the fusion protein is used as an antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. (See, D. Bennett *et al.*, *J. Molecular Recognition* 8:52-58 (1995); K. Johanson *et al.*, *J. Biol. Chem.* 270:9459-9471 (1995).)

Moreover, the disclosed angiogenic polypeptides can be fused to marker sequences, such as a peptide which facilitates purification of a particular angiogenic polypeptide. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA* 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Another peptide tag useful for purification, the "HA" tag, corresponds to an epitope derived from the influenza hemagglutinin protein. (Wilson *et al.*, *Cell* 37:767 (1984).)

Thus, any of these above fusions can be engineered using the disclosed angiogenic polynucleotides or the polypeptides.

#### **Vectors and Host Cells**

The present invention also relates to vectors containing polynucleotides encoding the disclosed angiogenic proteins, host cells, and the production of polypeptides by recombinant techniques. The vector may be, for example, a phage, plasmid, viral, or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

The polynucleotides encoding the disclosed angiogenic proteins may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged in vitro using an appropriate packaging cell line and then transduced into host cells.

The polynucleotide insert encoding the disclosed angiogenic protein of interest should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the E. coli lac, trp, phoA and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be



translated.

As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, 293, and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from QIAGEN, Inc.; pBluescript vectors, Phagescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene Cloning Systems, Inc.; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia Biotech, Inc. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., *Basic Methods In Molecular Biology* (1986). It is specifically contemplated that the disclosed angiogenic polypeptides may in fact be expressed by a host cell lacking a recombinant vector.

The disclosed angiogenic polypeptides can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

The disclosed angiogenic polypeptides, and preferably the secreted form, can also be recovered from: products purified from natural sources, including bodily fluids, tissues and cells, whether directly isolated or cultured; products of chemical synthetic procedures; and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect, and mammalian cells. Depending upon the host employed in a recombinant production procedure, the disclosed angiogenic polypeptides may be glycosylated or may be non-

glycosylated. In addition, the disclosed angiogenic polypeptides may also include an initial modified methionine residue, in some cases as a result of host-mediated processes. Thus, it is well known in the art that the N-terminal methionine encoded by the translation initiation codon generally is removed with high efficiency from any protein after translation in all eukaryotic cells. While the N-terminal methionine on most proteins also is efficiently removed in most prokaryotes, for some proteins, this prokaryotic removal process is inefficient, depending on the nature of the amino acid to which the N-terminal methionine is covalently linked.

In addition to encompassing host cells containing the vector constructs discussed herein, the invention also encompasses primary, secondary, and immortalized host cells of vertebrate origin, particularly mammalian origin, that have been engineered to delete or replace endogenous genetic material (e.g., angiogenic protein coding sequence), and/or to include genetic material (e.g., heterologous polynucleotide sequences) that is operably associated with polynucleotides encoding the disclosed angiogenic proteins of the invention, and which activates, alters, and/or amplifies endogenous polynucleotides encoding the disclosed angiogenic proteins. For example, techniques known in the art may be used to operably associate heterologous control regions (e.g., promoter and/or enhancer) and endogenous polynucleotides encoding the disclosed angiogenic protein sequences via homologous recombination (see, e.g., U.S. Patent No. 5,641,670, issued June 24, 1997; International Publication No. WO 96/29411, published September 26, 1996; International Publication No. WO 94/12650, published August 4, 1994; Koller et al., Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); and Zijlstra et al., Nature 342:435-438 (1989), the disclosures of each of which are incorporated by reference in their entireties).

In addition, polypeptides of the invention can be chemically synthesized using techniques known in the art (e.g., see Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y., and Hunkapiller, M., et al., 1984, Nature 310:105-111). For example, a peptide corresponding to a fragment of an angiogenic polypeptide of the invention can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the polynucleotide sequence encoding the angiogenic protein. Non-classical amino acids include, but are not limited to, to the D-isomers of the common amino acids, 2,4-diaminobutyric acid,  $\alpha$ -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid,  $\gamma$ -Abu,  $\epsilon$ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine,  $\beta$ -alanine, fluoro-amino

acids, designer amino acids such as  $\beta$ -methyl amino acids,  $\alpha$ -methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

The invention encompasses angiogenic polypeptides which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease,  $\text{NaBH}_4$ ; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

Additional post-translational modifications encompassed by the invention include, for example, *e.g.*, N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of procaryotic host cell expression. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

Also provided by the invention are chemically modified derivatives of angiogenic proteins which may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (see U. S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 1 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (*e.g.*, the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art, e.g., EP 0 401 384, herein incorporated by reference (coupling PEG to G-CSF), see also  
5 Malik et al., Exp. Hematol. 20:1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine  
10 residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecules. Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

15 One may specifically desire proteins chemically modified at the N-terminus. Using polyethylene glycol as an illustration of the present composition, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of  
20 obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective proteins chemically modified at the N-terminus modification may be accomplished by reductive  
25 alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved.

The angiogenic polypeptides of the invention may be in monomers or multimers  
30 (i.e., dimers, trimers, tetramers and higher multimers). Accordingly, the present invention relates to monomers and multimers of the disclosed angiogenic polypeptides of the invention, their preparation, and compositions (preferably, pharmaceutical compositions) containing them. In specific embodiments, the polypeptides of the invention are monomers, dimers, trimers or tetramers. In additional embodiments, the  
35 multimers of the invention are at least dimers, at least trimers, or at least tetramers.

Multimers encompassed by the invention may be homomers or heteromers. As used herein, the term homomer, refers to a multimer containing only angiogenic

polypeptides of the invention (including angiogenic protein fragments, variants, splice variants, and fusion proteins, as described herein). These homomers may contain angiogenic polypeptides having identical or different amino acid sequences. In a specific embodiment, a homomer of the invention is a multimer containing only polypeptides  
5 having an identical amino acid sequence of one of the disclosed angiogenic proteins. In another specific embodiment, a homomer of the invention is a multimer containing angiogenic polypeptides having different amino acid sequences. In specific embodiments, the multimer of the invention is a homodimer (e.g., containing angiogenic polypeptides having identical or different amino acid sequences) or a homotrimer (e.g.,  
10 containing angiogenic polypeptides having identical and/or different amino acid sequences). In additional embodiments, the homomeric multimer of the invention is at least a homodimer, at least a homotrimer, or at least a homotetramer.

As used herein, the term heteromer refers to a multimer containing one or more heterologous polypeptides (i.e., polypeptides of different proteins) in addition to the  
15 angiogenic polypeptides of the invention. In a specific embodiment, the multimer of the invention is a heterodimer, a heterotrimer, or a heterotetramer. In additional embodiments, the homomeric multimer of the invention is at least a homodimer, at least a homotrimer, or at least a homotetramer.

Multimers of the invention may be the result of hydrophobic, hydrophilic, ionic  
20 and/or covalent associations and/or may be indirectly linked, by for example, liposome formation. Thus, in one embodiment, multimers of the invention, such as, for example, homodimers or homotrimers, are formed when polypeptides of the invention contact one another in solution. In another embodiment, heteromultimers of the invention, such as, for example, heterotrimers or heterotetramers, are formed when polypeptides of the  
25 invention contact antibodies to the polypeptides of the invention (including antibodies to the heterologous polypeptide sequence in a fusion protein of the invention) in solution. In other embodiments, multimers of the invention are formed by covalent associations with and/or between the angiogenic polypeptides of the invention. Such covalent associations may involve one or more amino acid residues contained in the polypeptide  
30 sequences (e.g., that recited in SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24). In one instance, the covalent associations are cross-linking between cysteine residues located within the polypeptide sequences which interact in the native (i.e., naturally occurring) polypeptide. In another instance, the covalent associations are the consequence of chemical or recombinant manipulation. Alternatively, such covalent  
35 associations may involve one or more amino acid residues contained in the heterologous polypeptide sequence in an angiogenic fusion protein. In one example, covalent associations are between the heterologous sequence contained in a fusion protein of the

invention (see, e.g., US Patent Number 5,478,925). In a specific example, the covalent associations are between the heterologous sequence contained in an angiogenic polypeptide-Fc fusion protein of the invention (as described herein). In another specific example, covalent associations of fusion proteins of the invention are between  
5 heterologous polypeptide sequence from another TNF family ligand/receptor member that is capable of forming covalently associated multimers, such as for example, osteoprotegerin (see, e.g., International Publication No. WO 98/49305, the contents of which are herein incorporated by reference in its entirety).

The multimers of the invention may be generated using chemical techniques  
10 known in the art. For example, polypeptides desired to be contained in the multimers of the invention may be chemically cross-linked using linker molecules and linker molecule length optimization techniques known in the art (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally,  
15 multimers of the invention may be generated using techniques known in the art to form one or more inter-molecule cross-links between the cysteine residues located within the sequence of the polypeptides desired to be contained in the multimer (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Further, polypeptides of the invention may be routinely modified by the addition of cysteine or biotin to the C terminus or N-terminus of the polypeptide and techniques  
20 known in the art may be applied to generate multimers containing one or more of these modified polypeptides (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally, techniques known in the art may be applied to generate liposomes containing the polypeptide components desired to be contained in the multimer of the invention (see, e.g., US Patent Number 5,478,925,  
25 which is herein incorporated by reference in its entirety).

Alternatively, multimers of the invention may be generated using genetic engineering techniques known in the art. In one embodiment, polypeptides contained in multimers of the invention are produced recombinantly using fusion protein technology described herein or otherwise known in the art (see, e.g., US Patent Number 5,478,925,  
30 which is herein incorporated by reference in its entirety). In a specific embodiment, polynucleotides coding for a homodimer of the invention are generated by ligating a polynucleotide sequence encoding a polypeptide of the invention to a sequence encoding a linker polypeptide and then further to a synthetic polynucleotide encoding the translated product of the polypeptide in the reverse orientation from the original C-terminus to the  
35 N-terminus (lacking the leader sequence) (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). In another embodiment, recombinant techniques described herein or otherwise known in the art are applied to

generate recombinant polypeptides of the invention which contain a transmembrane domain (or hydrophobic or signal peptide) and which can be incorporated by membrane reconstitution techniques into liposomes (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety).

### ***Biological Activities of Angiogenic Polypeptides***

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, can be used in assays to test for one or more biological activities. If angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, do exhibit activity in a particular assay, it is likely that angiogenic polynucleotides or polypeptides may be involved in the diseases associated with the biological activity. Therefore, angiogenic polypeptides could be used to treat the associated disease. The following described biological activities are included within the angiogenic proteins "desired activities".

### **Immune Activity**

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may be useful in treating deficiencies or disorders of the immune system, by activating or inhibiting the proliferation, differentiation, or mobilization (chemotaxis) of immune cells. Immune cells develop through a process called hematopoiesis, producing myeloid (platelets, red blood cells, neutrophils, and macrophages) and lymphoid (B and T lymphocytes) cells from pluripotent stem cells. The etiology of these immune deficiencies or disorders may be genetic, somatic, such as cancer or some autoimmune disorders, acquired (e.g., by chemotherapy or toxins), or infectious. Moreover, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, can be used as a marker or detector of a particular immune system disease or disorder.

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may be useful in treating or detecting deficiencies or disorders of hematopoietic cells. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used to increase differentiation and proliferation of hematopoietic cells, including the pluripotent stem cells, in an effort to treat those disorders associated with a decrease in certain (or many) types hematopoietic cells. Examples of immunologic deficiency syndromes include, but are not limited to: blood protein disorders (e.g. agammaglobulinemia, dysgammaglobulinemia), ataxia telangiectasia, common variable immunodeficiency, DiGeorge Syndrome, HIV infection, HTLV-BLV infection, leukocyte

adhesion deficiency syndrome, lymphopenia, phagocyte bactericidal dysfunction, severe combined immunodeficiency (SCIDs), Wiskott-Aldrich Disorder, anemia, thrombocytopenia, or hemoglobinuria.

Moreover, angiogenic polynucleotides or polypeptides, or agonists or antagonists  
5 of angiogenic polynucleotides or polypeptides, can also be used to modulate hemostatic (the stopping of bleeding) or thrombolytic activity (clot formation). For example, by increasing hemostatic or thrombolytic activity, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used to treat blood coagulation disorders (e.g., afibrinogenemia, factor  
10 deficiencies), blood platelet disorders (e.g. thrombocytopenia), or wounds resulting from trauma, surgery, or other causes. Alternatively, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, that can decrease hemostatic or thrombolytic activity could be used to inhibit or dissolve clotting, important in the treatment of heart attacks (infarction), strokes, or scarring.

15 Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may also be useful in treating or detecting autoimmune disorders. Many autoimmune disorders result from inappropriate recognition of self as foreign material by immune cells. This inappropriate recognition results in an immune response leading to the destruction of the host tissue. Therefore, the  
20 administration of angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, that can inhibit an immune response, particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing autoimmune disorders.

Examples of autoimmune disorders that can be treated or detected include, but are  
25 not limited to: Addison's Disease, hemolytic anemia, antiphospholipid syndrome, rheumatoid arthritis, dermatitis, allergic encephalomyelitis, glomerulonephritis, Goodpasture's Syndrome, Graves' Disease, Multiple Sclerosis, Myasthenia Gravis, Neuritis, Ophthalmia, Bullous Pemphigoid, Pemphigus, Polyendocrinopathies, Purpura, Reiter's Disease, Stiff-Man Syndrome, Autoimmune Thyroiditis, Systemic Lupus  
30 Erythematosus, Autoimmune Pulmonary Inflammation, Guillain-Barre Syndrome, insulin dependent diabetes mellitus, and autoimmune inflammatory eye disease.

Similarly, allergic reactions and conditions, such as asthma (particularly allergic  
asthma) or other respiratory problems, may also be treated by angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or  
35 polypeptides. Moreover, these molecules can be used to treat anaphylaxis, hypersensitivity to an antigenic molecule, or blood group incompatibility.



Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may also be used to treat and/or prevent organ rejection or graft-versus-host disease (GVHD). Organ rejection occurs by host immune cell destruction of the transplanted tissue through an immune response.

5 Similarly, an immune response is also involved in GVHD, but, in this case, the foreign transplanted immune cells destroy the host tissues. The administration of angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, that inhibits an immune response, particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing organ  
10 rejection or GVHD.

Similarly, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may also be used to modulate inflammation. For example, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may inhibit the proliferation  
15 and differentiation of cells involved in an inflammatory response. These molecules can be used to treat inflammatory conditions, both chronic and acute conditions, including inflammation associated with infection (e.g., septic shock, sepsis, or systemic inflammatory response syndrome (SIRS)), ischemia-reperfusion injury, endotoxin lethality, arthritis, complement-mediated hyperacute rejection, nephritis, cytokine or  
20 chemokine induced lung injury, inflammatory bowel disease, Crohn's disease, or resulting from over production of cytokines (e.g., TNF or IL-1.)

### **Hyperproliferative Disorders**

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of  
25 angiogenic polynucleotides or polypeptides, can be used to treat or detect hyperproliferative disorders, including neoplasms. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may inhibit the proliferation of the disorder through direct or indirect interactions. Alternatively, angiogenic polynucleotides or polypeptides, or agonists or antagonists of  
30 angiogenic polynucleotides or polypeptides, may proliferate other cells which can inhibit the hyperproliferative disorder.

For example, by increasing an immune response, particularly increasing antigenic qualities of the hyperproliferative disorder or by proliferating, differentiating, or mobilizing T-cells, hyperproliferative disorders can be treated. This immune response  
35 may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, decreasing an immune response may also be a method of treating hyperproliferative disorders, such as a chemotherapeutic agent. Examples of

hyperproliferative disorders that can be treated or detected by angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, include, but are not limited to neoplasms located in the: abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic, and urogenital.

Similarly, other hyperproliferative disorders can also be treated or detected by angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides. Examples of such hyperproliferative disorders include, but are not limited to: hypergammaglobulinemia, lymphoproliferative disorders, paraproteinemias, purpura, sarcoidosis, Sezary Syndrome, Waldenstrom's Macroglobulinemia, Gaucher's Disease, histiocytosis, and any other hyperproliferative disease, besides neoplasia, located in an organ system listed above.

#### Cardiovascular Disorders

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides may be used to treat cardiovascular disorders, including peripheral artery disease, such as limb ischemia.

Cardiovascular disorders include cardiovascular abnormalities, such as arterio-arterial fistula, arteriovenous fistula, cerebral arteriovenous malformations, congenital heart defects, pulmonary atresia, and Scimitar Syndrome. Congenital heart defects include aortic coarctation, cor triatriatum, coronary vessel anomalies, crisscross heart, dextrocardia, patent ductus arteriosus, Ebstein's anomaly, Eisenmenger complex, hypoplastic left heart syndrome, levocardia, tetralogy of fallot, transposition of great vessels, double outlet right ventricle, tricuspid atresia, persistent truncus arteriosus, and heart septal defects, such as aortopulmonary septal defect, endocardial cushion defects, Lutembacher's Syndrome, trilogly of Fallot, ventricular heart septal defects.

Cardiovascular disorders also include heart disease, such as arrhythmias, carcinoid heart disease, high cardiac output, low cardiac output, cardiac tamponade, endocarditis (including bacterial), heart aneurysm, cardiac arrest, congestive heart failure, congestive cardiomyopathy, paroxysmal dyspnea, cardiac edema, heart hypertrophy, congestive cardiomyopathy, left ventricular hypertrophy, right ventricular hypertrophy, post-infarction heart rupture, ventricular septal rupture, heart valve diseases, myocardial diseases, myocardial ischemia, pericardial effusion, pericarditis (including constrictive and tuberculous), pneumopericardium, postpericardiotomy syndrome, pulmonary heart disease, rheumatic heart disease, ventricular dysfunction, hyperemia, cardiovascular

pregnancy complications, Scimitar Syndrome, cardiovascular syphilis, and cardiovascular tuberculosis.

Arrhythmias include sinus arrhythmia, atrial fibrillation, atrial flutter, bradycardia, extrasystole, Adams-Stokes Syndrome, bundle-branch block, sinoatrial block, long QT syndrome, parasystole, Lown-Ganong-Levine Syndrome, Mahaim-type pre-excitation syndrome, Wolff-Parkinson-White syndrome, sick sinus syndrome, tachycardias, and ventricular fibrillation. Tachycardias include paroxysmal tachycardia, supraventricular tachycardia, accelerated idioventricular rhythm, atrioventricular nodal reentry tachycardia, ectopic atrial tachycardia, ectopic junctional tachycardia, sinoatrial nodal reentry tachycardia, sinus tachycardia, Torsades de Pointes, and ventricular tachycardia.

Heart valve disease include aortic valve insufficiency, aortic valve stenosis, heart murmurs, aortic valve prolapse, mitral valve prolapse, tricuspid valve prolapse, mitral valve insufficiency, mitral valve stenosis, pulmonary atresia, pulmonary valve insufficiency, pulmonary valve stenosis, tricuspid atresia, tricuspid valve insufficiency, and tricuspid valve stenosis.

Myocardial diseases include alcoholic cardiomyopathy, congestive cardiomyopathy, hypertrophic cardiomyopathy, aortic subvalvular stenosis, pulmonary subvalvular stenosis, restrictive cardiomyopathy, Chagas cardiomyopathy, endocardial fibroelastosis, endomyocardial fibrosis, Kearns Syndrome, myocardial reperfusion injury, and myocarditis.

Myocardial ischemias include coronary disease, such as angina pectoris, coronary aneurysm, coronary arteriosclerosis, coronary thrombosis, coronary vasospasm, myocardial infarction and myocardial stunning.

Cardiovascular diseases also include vascular diseases such as aneurysms, angiodyplasia, angiomatosis, bacillary angiomatosis, Hippel-Lindau Disease, Klippel-Trenaunay-Weber Syndrome, Sturge-Weber Syndrome, angioneurotic edema, aortic diseases, Takayasu's Arteritis, aortitis, Leriche's Syndrome, arterial occlusive diseases, arteritis, enarteritis, polyarteritis nodosa, cerebrovascular disorders, diabetic angiopathies, diabetic retinopathy, embolisms, thrombosis, erythromelalgia, hemorrhoids, hepatic veno-occlusive disease, hypertension, hypotension, ischemia, peripheral vascular diseases, phlebitis, pulmonary veno-occlusive disease, Raynaud's disease, CREST syndrome, retinal vein occlusion, Scimitar syndrome, superior vena cava syndrome, telangiectasia, ataxia telangiectasia, hereditary hemorrhagic telangiectasia, varicocele, varicose veins, varicose ulcer, vasculitis, and venous insufficiency.

Aneurysms include dissecting aneurysms, false aneurysms, infected aneurysms, ruptured aneurysms, aortic aneurysms, cerebral aneurysms, coronary aneurysms, heart aneurysms, and iliac aneurysms.

Arterial occlusive diseases include arteriosclerosis, intermittent claudication, carotid stenosis, fibromuscular dysplasias, mesenteric vascular occlusion, Moyamoya disease, renal artery obstruction, retinal artery occlusion, and thromboangiitis obliterans.

Cerebrovascular disorders include carotid artery diseases, cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformation, cerebral artery diseases, cerebral embolism and thrombosis, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, cerebral hemorrhage, epidural hematoma, subdural hematoma, subarachnoid hemorrhage, cerebral infarction, cerebral ischemia (including transient), subclavian steal syndrome, periventricular leukomalacia, vascular headache, cluster headache, migraine, and vertebrobasilar insufficiency.

Embolisms include air embolisms, amniotic fluid embolisms, cholesterol embolisms, blue toe syndrome, fat embolisms, pulmonary embolisms, and thromboembolisms. Thrombosis include coronary thrombosis, hepatic vein thrombosis, retinal vein occlusion, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, and thrombophlebitis.

Ischemia includes cerebral ischemia, ischemic colitis, compartment syndromes, anterior compartment syndrome, myocardial ischemia, reperfusion injuries, and peripheral limb ischemia. Vasculitis includes aortitis, arteritis, Behcet's Syndrome, Churg-Strauss Syndrome, mucocutaneous lymph node syndrome, thromboangiitis obliterans, hypersensitivity vasculitis, Schoenlein-Henoch purpura, allergic cutaneous vasculitis, and Wegener's granulomatosis.

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, are especially effective for the treatment of critical limb ischemia and coronary disease. As shown in the Examples, administration of angiogenic polynucleotides and polypeptides to an experimentally induced ischemia rabbit hindlimb may restore blood pressure ratio, blood flow, angiographic score, and capillary density.

Angiogenic polypeptides may be administered using any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous injection, topical administration, catheter infusion, biolistic injectors, particle accelerators, gelfoam sponge depots, other commercially available depot materials, osmotic pumps, oral or suppository solid pharmaceutical formulations, decanting or topical applications during surgery, aerosol delivery. Such methods are known in the art. Angiogenic polypeptides may be administered as part of a pharmaceutical composition, described in more detail below. Methods of delivering angiogenic polynucleotides are described in more detail herein.

### Anti-Angiogenesis Activity

The naturally occurring balance between endogenous stimulators and inhibitors of angiogenesis is one in which inhibitory influences predominate. Rastinejad *et al.*, *Cell* 56:345-355 (1989). In those rare instances in which neovascularization occurs under normal physiological conditions, such as wound healing, organ regeneration, embryonic development, and female reproductive processes, angiogenesis is stringently regulated and spatially and temporally delimited. Under conditions of pathological angiogenesis such as that characterizing solid tumor growth, these regulatory controls fail. Unregulated angiogenesis becomes pathologic and sustains progression of many neoplastic and non-neoplastic diseases. A number of serious diseases are dominated by abnormal neovascularization including solid tumor growth and metastases, arthritis, some types of eye disorders, and psoriasis. See, e.g., reviews by Moses *et al.*, *Biotech.* 9:630-634 (1991); Folkman *et al.*, *N. Engl. J. Med.*, 333:1757-1763 (1995); Auerbach *et al.*, *J. Microvasc. Res.* 29:401-411 (1985); Folkman, *Advances in Cancer Research*, eds. Klein and Weinhouse, Academic Press, New York, pp. 175-203 (1985); Patz, *Am. J. Ophthalmol.* 94:715-743 (1982); and Folkman *et al.*, *Science* 221:719-725 (1983). In a number of pathological conditions, the process of angiogenesis contributes to the disease state. For example, significant data have accumulated which suggest that the growth of solid tumors is dependent on angiogenesis. Folkman and Klagsbrun, *Science* 235:442-447 (1987).

The present invention provides for treatment of diseases or disorders associated with neovascularization by administration of the angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides. Malignant and metastatic conditions which can be treated with the polynucleotides and polypeptides, or agonists or antagonists of the invention include, but are not limited to, malignancies, solid tumors, and cancers described herein and otherwise known in the art (for a review of such disorders, see Fishman *et al.*, *Medicine*, 2d Ed., J. B. Lippincott Co., Philadelphia (1985)):

Ocular disorders associated with neovascularization which can be treated with the angiogenic polynucleotides and polypeptides of the present invention (including angiogenic agonists and/or antagonists) include, but are not limited to: neovascular glaucoma, diabetic retinopathy, retinoblastoma, retrolental fibroplasia, uveitis, retinopathy of prematurity macular degeneration, corneal graft neovascularization, as well as other eye inflammatory diseases, ocular tumors and diseases associated with choroidal or iris neovascularization. See, e.g., reviews by Waltman *et al.*, *Am. J. Ophthalm.* 85:704-710 (1978) and Gartner *et al.*, *Surv. Ophthalm.* 22:291-312 (1978).

Additionally, disorders which can be treated with the angiogenic polynucleotides and polypeptides of the present invention (including angiogenic agonist and/or antagonists) include, but are not limited to, hemangioma, arthritis, psoriasis, angiofibroma, atherosclerotic plaques, delayed wound healing, granulations, hemophilic joints, hypertrophic scars, nonunion fractures, Osler-Weber syndrome, pyogenic granuloma, scleroderma, trachoma, and vascular adhesions.

Moreover, disorders and/or states, which can be treated with the angiogenic polynucleotides and polypeptides of the present invention (including angiogenic agonist and/or antagonists) include, but are not limited to, solid tumors, blood born tumors such as leukemias, tumor metastasis, Kaposi's sarcoma, benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas, rheumatoid arthritis, psoriasis, ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, and uveitis, delayed wound healing, endometriosis, vasculogenesis, granulations, hypertrophic scars (keloids), nonunion fractures, scleroderma, trachoma, vascular adhesions, myocardial angiogenesis, coronary collaterals, cerebral collaterals, arteriovenous malformations, ischemic limb angiogenesis, Osler-Webber Syndrome, plaque neovascularization, telangiectasia, hemophilic joints, angiofibroma fibromuscular dysplasia, wound granulation, Crohn's disease, atherosclerosis, birth control agent by preventing vascularization required for embryo implantation controlling menstruation, diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (*Rochela minialia quintosa*), ulcers (*Helicobacter pylori*), Bartonellosis and bacillary angiomatosis.

#### **Diseases at the Cellular Level**

Diseases associated with increased cell survival or the inhibition of apoptosis that could be treated or detected by angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, including, but not limited to colon cancer, cardiac tumors, pancreatic cancer, melanoma, retinoblastoma, glioblastoma, lung cancer, intestinal cancer, testicular cancer, stomach cancer, neuroblastoma, myxoma, myoma, lymphoma, endothelioma, osteoblastoma, osteoclastoma, osteosarcoma, chondrosarcoma, adenoma, breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-

related glomerulonephritis and rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), inflammation, graft v. host disease, acute graft rejection, and chronic graft rejection. In preferred embodiments, angiogenic polynucleotides, polypeptides, and/or antagonists of the invention are used to inhibit growth, progression, and/or metasis of cancers, in particular those listed above.

Additional diseases or conditions associated with increased cell survival that could be treated or detected by angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (e.g., acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (e.g., chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (e.g., Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma, and retinoblastoma.

Diseases associated with increased apoptosis that could be treated or detected by angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration and brain tumor or prior associated disease); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) myelodysplastic syndromes (such as aplastic anemia), graft v. host disease, ischemic

injury (such as that caused by myocardial infarction, stroke and reperfusion injury), liver injury (e.g., hepatitis related liver injury, ischemia/reperfusion injury, cholestosis (bile duct injury) and liver cancer); toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

5

### **Wound Healing and Epithelial Cell Proliferation**

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, for therapeutic purposes, for  
10 example, to stimulate epithelial cell proliferation and basal keratinocytes for the purpose of wound healing, and to stimulate hair follicle production and healing of dermal wounds. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may be clinically useful in stimulating wound healing including surgical wounds, excisional wounds, deep wounds involving  
15 damage of the dermis and epidermis, eye tissue wounds, dental tissue wounds, oral cavity wounds, diabetic ulcers, dermal ulcers, cubitus ulcers, arterial ulcers, venous stasis ulcers, burns resulting from heat exposure or chemicals, and other abnormal wound healing conditions such as uremia, malnutrition, vitamin deficiencies and complications associated with systemic treatment with steroids, radiation therapy and  
20 antineoplastic drugs and antimetabolites. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used to promote dermal reestablishment subsequent to dermal loss

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used to increase the adherence of  
25 skin grafts to a wound bed and to stimulate re-epithelialization from the wound bed. The following are types of grafts that angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used to increase adherence to a wound bed: autografts, artificial skin, allografts, autodermic graft, autoepdermic grafts, avacular grafts, Blair-Brown grafts, bone graft, brephoplastic  
30 grafts, cutis graft, delayed graft, dermic graft, epidermic graft, fascia graft, full thickness graft, heterologous graft, xenograft, homologous graft, hyperplastic graft, lamellar graft, mesh graft, mucosal graft, Ollier-Thiersch graft, omenpal graft, patch graft, pedicle graft, penetrating graft, split skin graft, thick split graft. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides,  
35 can be used to promote skin strength and to improve the appearance of aged skin.

It is believed that angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, will also produce changes in



hepatocyte proliferation, and epithelial cell proliferation in the lung, breast, pancreas, stomach, small intestine, and large intestine. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could promote proliferation of epithelial cells such as sebocytes, hair follicles, hepatocytes, type II pneumocytes, mucin-producing goblet cells, and other epithelial cells and their progenitors contained within the skin, lung, liver, and gastrointestinal tract. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may promote proliferation of endothelial cells, keratinocytes, and basal keratinocytes.

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could also be used to reduce the side effects of gut toxicity that result from radiation, chemotherapy treatments or viral infections. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may have a cytoprotective effect on the small intestine mucosa. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may also stimulate healing of mucositis (mouth ulcers) that result from chemotherapy and viral infections.

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could further be used in full regeneration of skin in full and partial thickness skin defects, including burns, (i.e., repopulation of hair follicles, sweat glands, and sebaceous glands), treatment of other skin defects such as psoriasis. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used to treat epidermolysis bullosa, a defect in adherence of the epidermis to the underlying dermis which results in frequent, open and painful blisters by accelerating reepithelialization of these lesions. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could also be used to treat gastric and duodenal ulcers and help heal by scar formation of the mucosal lining and regeneration of glandular mucosa and duodenal mucosal lining more rapidly. Inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, are diseases which result in destruction of the mucosal surface of the small or large intestine, respectively. Thus, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used to promote the resurfacing of the mucosal surface to aid more rapid healing and to prevent progression of inflammatory bowel disease. Treatment with angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, is expected to have a significant effect on the production of mucus throughout the gastrointestinal tract and could be used to protect the intestinal mucosa

from injurious substances that are ingested or following surgery. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used to treat diseases associate with the under expression of angiogenic polypeptides.

5        Moreover, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used to prevent and heal damage to the lungs due to various pathological states. A growth factor such as angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, which could stimulate proliferation and differentiation and promote the  
10        repair of alveoli and brochiolar epithelium to prevent or treat acute or chronic lung damage. For example, emphysema, which results in the progressive loss of aveoli, and inhalation injuries, i.e., resulting from smoke inhalation and burns, that cause necrosis of the bronchiolar epithelium and alveoli could be effectively treated using angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides  
15        or polypeptides. Also, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used to stimulate the proliferation of and differentiation of type II pneumocytes, which may help treat or prevent disease such as hyaline membrane diseases, such as infant respiratory distress syndrome and bronchopulmonary displasia, in premature infants.

20        Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could stimulate the proliferation and differentiation of hepatocytes and, thus, could be used to alleviate or treat liver diseases and pathologies such as fulminant liver failure caused by cirrhosis, liver damage caused by viral hepatitis and toxic substances (i.e., acetaminophen, carbon tetraholoride and  
25        other hepatotoxins known in the art).

      In addition, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used treat or prevent the onset of diabetes mellitus. In patients with newly diagnosed Types I and II diabetes, where some islet cell function remains, angiogenic polynucleotides or polypeptides, or agonists or  
30        antagonists of angiogenic polynucleotides or polypeptides, could be used to maintain the islet function so as to alleviate, delay or prevent permanent manifestation of the disease. Also, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used as an auxiliary in islet cell transplantation to improve or promote islet cell function.

**Infectious Disease**

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, can be used to treat or detect infectious agents. For example, by increasing the immune response, particularly increasing the proliferation and differentiation of B and/or T cells, infectious diseases may be treated. The immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may also directly inhibit the infectious agent, without necessarily eliciting an immune response.

Viruses are one example of an infectious agent that can cause disease or symptoms that can be treated or detected by angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides. Examples of viruses, include, but are not limited to the following DNA and RNA viral families: Arbovirus, Adenoviridae, Arenaviridae, Arterivirus, Birnaviridae, Bunyaviridae, Caliciviridae, Circoviridae, Coronaviridae, Flaviviridae, Hepadnaviridae (Hepatitis), Herpesviridae (such as, Cytomegalovirus, Herpes Simplex, Herpes Zoster), Mononegavirus (e.g., Paramyxoviridae, Morbillivirus, Rhabdoviridae), Orthomyxoviridae (e.g., Influenza), Papovaviridae, Parvoviridae, Picornaviridae, Poxviridae (such as Smallpox or Vaccinia), Reoviridae (e.g., Rotavirus), Retroviridae (HTLV-I, HTLV-II, Lentivirus), and Togaviridae (e.g., Rubivirus). Viruses falling within these families can cause a variety of diseases or symptoms, including, but not limited to: arthritis, bronchiolitis, encephalitis, eye infections (e.g., conjunctivitis, keratitis), chronic fatigue syndrome, hepatitis (A, B, C, E, Chronic Active, Delta), meningitis, opportunistic infections (e.g., AIDS), pneumonia, Burkitt's Lymphoma, chickenpox, hemorrhagic fever, Measles, Mumps, Parainfluenza, Rabies, the common cold, Polio, leukemia, Rubella, sexually transmitted diseases, skin diseases (e.g., Kaposi's, warts), and viremia. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, can be used to treat or detect any of these symptoms or diseases.

Similarly, bacterial or fungal agents that can cause disease or symptoms and that can be treated or detected by angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, include, but not limited to, the following Gram-Negative and Gram-positive bacterial families and fungi: Actinomycetales (e.g., Corynebacterium, Mycobacterium, Norcardia), Aspergillosis, Bacillaceae (e.g., Anthrax, Clostridium), Bacteroidaceae, Blastomycosis, Bordetella, Borrelia, Brucellosis, Candidiasis, Campylobacter, Coccidioidomycosis,

Cryptococcosis, Dermatocycoses, Enterobacteriaceae (Klebsiella, Salmonella, Serratia, Yersinia), Erysipelothrix, Helicobacter, Legionellosis, Leptospirosis, Listeria, Mycoplasmatales, Neisseriaceae (e.g., Acinetobacter, Gonorrhea, Meningococcal), Pasteurellaceae Infections (e.g., Actinobacillus, Haemophilus, Pasteurella),  
5 Pseudomonas, Rickettsiaceae, Chlamydiaceae, Syphilis, and Staphylococcal. These bacterial or fungal families can cause the following diseases or symptoms, including, but not limited to: bacteremia, endocarditis, eye infections (conjunctivitis, tuberculosis, uveitis), gingivitis, opportunistic infections (e.g., AIDS related infections), paronychia, prosthesis-related infections, Reiter's Disease, respiratory tract infections, such as  
10 Whooping Cough or Empyema, sepsis, Lyme Disease, Cat-Scratch Disease, Dysentery, Paratyphoid Fever, food poisoning, Typhoid, pneumonia, Gonorrhea, meningitis, Chlamydia, Syphilis, Diphtheria, Leprosy, Paratuberculosis, Tuberculosis, Lupus, Botulism, gangrene, tetanus, impetigo, Rheumatic Fever, Scarlet Fever, sexually transmitted diseases, skin diseases (e.g., cellulitis, dermatocycoses), toxemia, urinary  
15 tract infections, wound infections. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, can be used to treat or detect any of these symptoms or diseases.

Moreover, parasitic agents causing disease or symptoms that can be treated or detected by angiogenic polynucleotides or polypeptides, or agonists or antagonists of  
20 angiogenic polynucleotides or polypeptides, include, but not limited to, the following families: Amebiasis, Babesiosis, Coccidiosis, Cryptosporidiosis, Dientamoebiasis, Dourine, Ectoparasitic, Giardiasis, Helminthiasis, Leishmaniasis, Theileriasis, Toxoplasmosis, Trypanosomiasis, and Trichomonas. These parasites can cause a variety of diseases or symptoms, including, but not limited to: Scabies, Trombiculiasis, eye  
25 infections, intestinal disease (e.g., dysentery, giardiasis), liver disease, lung disease, opportunistic infections (e.g., AIDS related), Malaria, pregnancy complications, and toxoplasmosis. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, can be used to treat or detect any of these symptoms or diseases.

30 Preferably, treatment using angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could either be by administering an effective amount of angiogenic polypeptide to the patient, or by removing cells from the patient, supplying the cells with angiogenic polynucleotide, and returning the engineered cells to the patient (ex vivo therapy). Moreover, the angiogenic  
35 polypeptide or polynucleotide can be used as an antigen in a vaccine to raise an immune response against infectious disease.

**Regeneration**

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, can be used to differentiate, proliferate, and attract cells, leading to the regeneration of tissues. (See, Science 276:59-87 (1997).)

- 5 The regeneration of tissues could be used to repair, replace, or protect tissue damaged by congenital defects, trauma (wounds, burns, incisions, or ulcers), age, disease (e.g. osteoporosis, osteoarthritis, periodontal disease, liver failure), surgery, including cosmetic plastic surgery, fibrosis, reperfusion injury, or systemic cytokine damage.

- 10 Tissues that could be regenerated using the present invention include organs (e.g., pancreas, liver, intestine, kidney, skin, endothelium), muscle (smooth, skeletal or cardiac), vasculature (including vascular and lymphatics), nervous, hematopoietic, and skeletal (bone, cartilage, tendon, and ligament) tissue. Preferably, regeneration occurs without or decreased scarring. Regeneration also may include angiogenesis.

- 15 Moreover, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may increase regeneration of tissues difficult to heal. For example, increased tendon/ligament regeneration would quicken recovery time after damage. Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, of the present invention could also be used prophylactically in an effort to avoid damage. Specific diseases that could  
20 be treated include of tendinitis, carpal tunnel syndrome, and other tendon or ligament defects. A further example of tissue regeneration of non-healing wounds includes pressure ulcers, ulcers associated with vascular insufficiency, surgical, and traumatic wounds.

- 25 Similarly, nerve and brain tissue could also be regenerated by using angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, to proliferate and differentiate nerve cells. Diseases that could be treated using this method include central and peripheral nervous system diseases, neuropathies, or mechanical and traumatic disorders (e.g., spinal cord disorders, head trauma, cerebrovascular disease, and stroke). Specifically, diseases associated with peripheral  
30 nerve injuries, peripheral neuropathy (e.g., resulting from chemotherapy or other medical therapies), localized neuropathies, and central nervous system diseases (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome), could all be treated using the angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides  
35 or polypeptides.

**Chemotaxis**

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may have chemotaxis activity. A chemotactic molecule attracts or mobilizes cells (e.g., monocytes, fibroblasts, neutrophils, T-cells, mast cells, eosinophils, epithelial and/or endothelial cells) to a particular site in the body, such as inflammation, infection, or site of hyperproliferation. The mobilized cells can then fight off and/or heal the particular trauma or abnormality.

Angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may increase chemotactic activity of particular cells. These chemotactic molecules can then be used to treat inflammation, infection, hyperproliferative disorders, or any immune system disorder by increasing the number of cells targeted to a particular location in the body. For example, chemotactic molecules can be used to treat wounds and other trauma to tissues by attracting immune cells to the injured location. As a chemotactic molecule, angiogenic polypeptides could also attract fibroblasts, which can be used to treat wounds.

It is also contemplated that angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, may inhibit chemotactic activity. These molecules could also be used to treat disorders. Thus, angiogenic polynucleotides or polypeptides, or agonists or antagonists of angiogenic polynucleotides or polypeptides, could be used as an inhibitor of chemotaxis.

**Binding Activity**

Angiogenic polypeptides may be used to screen for molecules that bind to angiogenic polypeptides or for molecules to which angiogenic polypeptides bind. The binding of an angiogenic polypeptide and the molecule may activate (agonist), increase, inhibit (antagonist), or decrease activity of the angiogenic polypeptide or the molecule bound. Examples of such molecules include antibodies, oligonucleotides, proteins (e.g., receptors), or small molecules.

Preferably, the screening for these molecules involves producing appropriate cells which express the angiogenic polypeptide, either as a secreted protein or on the cell membrane. Preferred cells include cells from mammals, yeast, *Drosophila*, or *E. coli*. Cells expressing the angiogenic polypeptide (or cell membrane containing the expressed polypeptide) are then preferably contacted with a test compound potentially containing the molecule to observe binding, stimulation, or inhibition of activity of either the angiogenic polypeptide or the molecule.

The assay may simply test binding of a candidate compound to the angiogenic polypeptide, wherein binding is detected by a label, or in an assay involving competition

with a labeled competitor. Further, the assay may test whether the candidate compound results in a signal generated by binding to the angiogenic polypeptide.

Alternatively, the assay can be carried out using cell-free preparations, polypeptide/molecule affixed to a solid support, chemical libraries, or natural product mixtures. The assay may also simply comprise the steps of mixing a candidate  
5 compound with a solution containing the angiogenic polypeptide, measuring angiogenic polypeptide/molecule activity or binding, and comparing the angiogenic polypeptide/molecule activity or binding to a standard.

Preferably, an ELISA assay can measure angiogenic polypeptide level or activity  
10 in a sample (e.g., biological sample) using a monoclonal or polyclonal antibody. The antibody can measure angiogenic polypeptide level or activity by either binding, directly or indirectly, to the angiogenic polypeptide or by competing with the angiogenic polypeptide for a substrate.

Additionally, the receptor to which the angiogenic polypeptide binds can be  
15 identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). For example, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to the polypeptides, for example, NIH3T3 cells which are known to contain multiple receptors for the FGF family proteins, and SC-3 cells, and  
20 a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to the polypeptides. Transfected cells which are grown on glass slides are exposed to the polypeptide of the present invention, after they have been labelled. The polypeptides can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase.

Following fixation and incubation, the slides are subjected to auto-radiographic  
25 analysis. Positive pools are identified and sub-pools are prepared and re-transfected using an iterative sub-pooling and re-screening process, eventually yielding a single clones that encodes the putative receptor.

As an alternative approach for receptor identification, the labeled polypeptides can  
30 be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE analysis and exposed to X-ray film. The labeled complex containing the receptors of the polypeptides can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of  
35 degenerate oligonucleotide probes to screen a cDNA library to identify the genes encoding the putative receptors.

Additionally, this invention provides a method of screening compounds to identify those which modulate the action of the polypeptide of the present invention. An example of such an assay comprises combining a mammalian fibroblast cell, a the polypeptide of the present invention, the compound to be screened and  $^3\text{[H]}$  thymidine under cell culture conditions where the fibroblast cell would normally proliferate. A control assay may be performed in the absence of the compound to be screened and compared to the amount of fibroblast proliferation in the presence of the compound to determine if the compound stimulates proliferation by determining the uptake of  $^3\text{[H]}$  thymidine in each case. The amount of fibroblast cell proliferation is measured by liquid scintillation chromatography which measures the incorporation of  $^3\text{[H]}$  thymidine. Both agonist and antagonist compounds may be identified by this procedure.

In another method, a mammalian cell or membrane preparation expressing a receptor for a polypeptide of the present invention is incubated with a labeled polypeptide of the present invention in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured. Alternatively, the response of a known second messenger system following interaction of a compound to be screened and the angiogenic polypeptide receptor is measured and the ability of the compound to bind to the receptor and elicit a second messenger response is measured to determine if the compound is a potential agonist or antagonist. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis.

All of these above assays can be used as diagnostic or prognostic markers. The molecules discovered using these assays can be used to treat disease or to bring about a particular result in a patient (e.g., blood vessel growth) by activating or inhibiting the angiogenic polypeptide/molecule. Moreover, the assays can discover agents which may inhibit or enhance the production of the angiogenic polypeptide from suitably manipulated cells or tissues. Therefore, the invention includes a method of identifying compounds which bind to the angiogenic polypeptide comprising the steps of: (a) incubating a candidate binding compound with the angiogenic polypeptide; and (b) determining if binding has occurred. Moreover, the invention includes a method of identifying agonists/antagonists comprising the steps of: (a) incubating a candidate compound with the angiogenic polypeptide, (b) assaying a biological activity, and (b) determining if a biological activity of the angiogenic polypeptide has been altered.



**Antisense And Ribozyme (Antagonists)**

In specific embodiments, antagonists according to the present invention are nucleic acids corresponding to the sequences contained in SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23, or the complementary strand thereof. In one embodiment, antisense sequence is generated internally by the organism, in another embodiment, the antisense sequence is separately administered (see, for example, O'Connor, J., Neurochem. 56:560 (1991). Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Antisense technology can be used to control gene expression through antisense DNA or RNA, or through triple-helix formation. Antisense techniques are discussed for example, in Okano, J., Neurochem. 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance, Lee et al., Nucleic Acids Research 6:3073 (1979); Cooney et al., Science 241:456 (1988); and Dervan et al., Science 251:1300 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into receptor polypeptide.

In one embodiment, the angiogenic antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector or a portion thereof, is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the angiogenic antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in vertebrate cells. Expression of the sequence encoding angiogenic protein, or fragments thereof, can be by any promoter known in the art to act in vertebrate, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, Nature 29:304-310 (1981), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., Cell 22:797-797 (1980), the herpes thymidine promoter (Wagner et al., Proc. Natl.

Acad. Sci. U.S.A. 78:1441-1445 (1981), the regulatory sequences of the metallothionein gene (Brinster, et al., Nature 296:39-42 (1982)), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of an angiogenic gene. However, absolute  
5 complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double stranded antisense nucleic acids targeted against RNA transcripts encoding angiogenic proteins, a single strand of the duplex DNA may thus be tested, or triplex  
10 formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the larger the hybridizing nucleic acid, the more base mismatches with an angiogenic RNA it may contain and still form a stable duplex (or triplex as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to  
15 determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting  
20 translation of mRNAs as well. See generally, Wagner, R., 1994, Nature 372:333-335. Thus, oligonucleotides complementary to either the 5'- or 3'- non-translated, non-coding regions of angiogenic proteins shown in Figures 3A-3C could be used in an antisense approach to inhibit translation of endogenous mRNA encoding angiogenic proteins. Oligonucleotides complementary to the 5' untranslated region of the mRNA should  
25 include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5', 3'- or coding region of angiogenic mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50  
30 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

The polynucleotides of the invention can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate  
35 backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors in vivo), or agents facilitating transport across the cell membrane (see,

e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, e.g., Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents. (See, e.g., Zon, 1988, Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group including, but not limited to, a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an a-anomeric oligonucleotide. An a-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual b-units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

Polynucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

While antisense nucleotides complementary to the coding region sequence encoding angiogenic proteins could be used, those complementary to the transcribed untranslated region are most preferred.

Potential antagonists according to the invention also include catalytic RNA, or a ribozyme (See, e.g., PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al, Science 247:1222-1225 (1990). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy mRNAs encoding angiogenic proteins, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, Nature 334:585-591 (1988). There are numerous potential hammerhead ribozyme cleavage sites within the nucleotide sequences encoding angiogenic proteins (SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23). Preferably, the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the mRNA encoding the angiogenic protein; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

As in the antisense approach, the ribozymes of the invention can be composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express angiogenic proteins in vivo. DNA constructs encoding the ribozyme may be introduced into the cell in the same manner as described above for the introduction of antisense encoding DNA. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive promoter, such as, for example, pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous messages encoding angiogenic proteins and inhibit their translation. Since ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Antagonist/agonist compounds may be employed to inhibit the cell growth and proliferation effects of the polypeptides of the present invention on neoplastic cells and

tissues, i.e. stimulation of angiogenesis of tumors, and, therefore, retard or prevent abnormal cellular growth and proliferation, for example, in tumor formation or growth.

The antagonist/agonist may also be employed to prevent hyper-vascular diseases, and prevent the proliferation of epithelial lens cells after extracapsular cataract surgery.

5 Prevention of the mitogenic activity of the polypeptides of the present invention may also be desirous in cases such as restenosis after balloon angioplasty.

The antagonist/agonist may also be employed to prevent the growth of scar tissue during wound healing.

10 The antagonist/agonist may also be employed to treat the diseases described herein.

#### Other Activities

The polypeptides of the present invention, as a result of the ability to stimulate vascular endothelial cell growth, may be employed in treatment for stimulating re-vascularization of ischemic tissues due to various disease conditions such as thrombosis, arteriosclerosis, and other cardiovascular conditions. These polypeptides may also be employed to stimulate angiogenesis and limb regeneration, as discussed above.

20 The polypeptides may also be employed for treating wounds due to injuries, burns, post-operative tissue repair, and ulcers since it has the ability to be mitogenic to various cells of different origins, such as fibroblast cells and skeletal muscle cells, and therefore, facilitate the repair or replacement of damaged or diseased tissue.

The polypeptides of the present invention may also be employed to stimulate neuronal growth and to treat and prevent neuronal damage which occurs in certain neuronal disorders or neuro-degenerative conditions such as Alzheimer's disease, Parkinson's disease, and AIDS-related complex. Angiogenic proteins, and agonists and/or antagonists to angiogenic proteins, may have the ability to stimulate chondrocyte growth, therefore, they may be employed to enhance bone and periodontal regeneration and aid in tissue transplants or bone grafts.

30 The polypeptides of the present invention may be also be employed to prevent skin aging due to sunburn by stimulating keratinocyte growth.

Angiogenic proteins, and agonists and/or antagonists to angiogenic proteins, may also be employed to maintain organs before transplantation or for supporting cell culture of primary tissues.

35 The polypeptides of the present invention may also be employed for inducing tissue of mesodermal origin to differentiate in early embryos.

Angiogenic proteins, and agonists and/or antagonists to angiogenic proteins, may also increase or decrease the differentiation or proliferation of embryonic stem cells,

besides, as discussed above, hematopoietic lineage.

Angiogenic proteins, and agonists and/or antagonists to angiogenic proteins, may also be used to modulate mammalian characteristics, such as body height, weight, hair color, eye color, skin, percentage of adipose tissue, pigmentation, size, and shape (e.g.,  
5 cosmetic surgery). Similarly, angiogenic proteins, and agonists and/or antagonists to angiogenic proteins, may be used to modulate mammalian metabolism affecting catabolism, anabolism, processing, utilization, and storage of energy.

Angiogenic proteins, and agonists and/or antagonists to angiogenic proteins, may be used to change a mammal's mental state or physical state by influencing biorhythms, circadian rhythms, depression (including depressive disorders), tendency for violence,  
10 tolerance for pain, reproductive capabilities (preferably by Activin or Inhibin-like activity), hormonal or endocrine levels, appetite, libido, memory, stress, or other cognitive qualities.

Angiogenic proteins, and agonists and/or antagonists to angiogenic proteins, may also be used as a food additive or preservative, such as to increase or decrease storage  
15 capabilities, fat content, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional components.

The above-recited applications have uses in a wide variety of hosts. Such hosts include, but are not limited to, human, murine, rabbit, goat, guinea pig, camel, horse,  
20 mouse, rat, hamster, pig, micro-pig, chicken, goat, cow, sheep, dog, cat, non-human primate, and human. In specific embodiments, the host is a mouse, rabbit, goat, guinea pig, chicken, rat, hamster, pig, sheep, dog or cat. In preferred embodiments, the host is a mammal. In most preferred embodiments, the host is a human.

## 25 *Therapeutic Uses*

The angiogenic proteins of the present invention are potent mitogens for vascular and lymphatic endothelial cells. Accordingly, angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may be employed to treat vascular trauma by promoting angiogenesis. For example, to stimulate the  
30 growth of transplanted tissue where coronary bypass surgery is performed. Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be employed to promote wound healing, particularly to re-vascularize damaged tissues or stimulate collateral blood flow during ischemia and where new capillary angiogenesis is desired. Angiogenic polypeptides, or biologically active  
35 portions thereof, in conjunction with agonists and/or antagonists may be employed to treat full-thickness wounds such as dermal ulcers, including pressure sores, venous ulcers, and diabetic ulcers. In addition, angiogenic polypeptides, or biologically active

portions thereof, in conjunction with agonists and/or antagonists may be employed to treat full-thickness burns and injuries where a skin graft or flap is used to repair such burns and injuries. Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be employed for use in plastic surgery, for example, for the repair of lacerations, burns, or other trauma. In addition, angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists can be used to promote healing of wounds and injuries to the eye as well as to treat eye diseases.

Along these same lines, angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be employed to induce the growth of damaged bone, periodontium or ligament tissue. Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be employed for regenerating supporting tissues of the teeth, including cementum and periodontal ligament, that have been damaged by, *e.g.*, periodontal disease or trauma.

Since angiogenesis is important in keeping wounds clean and non-infected, angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may be employed in association with surgery and following the repair of incisions and cuts. Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be employed for the treatment of abdominal wounds where there is a high risk of infection.

Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may be employed for the promotion of endothelialization in vascular graft surgery. In the case of vascular grafts using either transplanted or synthetic material, angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists can be applied to the surface of the graft or at the junction to promote the growth of vascular endothelial cells. Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be employed to repair damage of myocardial tissue as a result of myocardial infarction. Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be employed to repair the cardiac vascular system after ischemia. Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be employed to treat damaged vascular tissue as a result of coronary artery disease and peripheral and CNS vascular disease.

Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be employed to coat artificial prostheses or

natural organs which are to be transplanted in the body to minimize rejection of the transplanted material and to stimulate vascularization of the transplanted materials.

Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be employed for vascular tissue repair of injuries resulting from trauma, for example, that occurring during arteriosclerosis and required following balloon angioplasty where vascular tissues are damaged.

Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be used to treat peripheral arterial disease. Accordingly, in a further aspect, there is provided a process for utilizing angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists to treat peripheral arterial disease. Preferably, an angiogenic polypeptide, co-treated with an agonist is administered to an individual for the purpose of alleviating or treating peripheral arterial disease. Suitable doses, formulations, and administration routes are described below.

Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be used to promote the endothelial function of lymphatic tissues and vessels, such as to treat the loss of lymphatic vessels, occlusions of lymphatic vessels, and lymphangiomas. Angiogenic polypeptides in conjunction with agonists may also be used to stimulate lymphocyte production.

Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be used to treat hemangioma in newborns. Accordingly, in a further aspect, there is provided a process for utilizing angiogenic polypeptides co-treated with an agonist and/or antagonist to treat hemangioma in newborns. Preferably, an angiogenic polypeptide, co-treated with an agonist is administered to an individual for the purpose of alleviating or treating hemangioma in newborns. Suitable doses, formulations, and administration routes are described below.

Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be used to prevent or treat abnormal retinal development in premature newborns. Accordingly, in a further aspect, there is provided a process for utilizing angiogenic polypeptides, in conjunction with either agonists or antagonists to treat abnormal retinal development in premature newborns. Preferably, an angiogenic polypeptide, co-treated with an agonist or an antagonist is administered to an individual for the purpose of alleviating or treating abnormal retinal development in premature newborns. Suitable doses, formulations, and administration routes are described below.

Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may be used to treat primary (idiopathic) lymphadenomas,



including Milroy's disease and Lymphedema praecox. Accordingly, in a further aspect, there is provided a process for utilizing angiogenic polypeptides in conjunction with an agonist to treat primary (idiopathic) lymphademas, including Milroy's disease and Lymphedema praecox. Preferably, an angiogenic polypeptide, co-treated with an agonist is administered to an individual for the purpose of alleviating or treating primary (idiopathic) lymphademas, including Milroy's disease and Lymphedema praecox. Angiogenic polypeptides in conjunction with agonists may also be used to treat edema as well as to effect blood pressure in an animal. Suitable doses, formulations, and administration routes are described below.

Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be used to treat secondary (obstructive) lifetimes including those that result from (I) the removal of lymph nodes and vessels, (ii) radiotherapy and surgery in the treatment of cancer, and (iii) trauma and infection. Accordingly, in a further aspect, there is provided a process for utilizing angiogenic polypeptides in conjunction with agonists to treat secondary (obstructive) lifetimes including those that result from (I) the removal of lymph nodes and vessels, (ii) radiotherapy and surgery in the treatment of cancer, and (iii) trauma and infection. Preferably, an angiogenic polypeptide is co-administered with an agonist to an individual for the purpose of secondary (obstructive) lifetimes including those that result from (I) the removal of lymph nodes and vessels, (ii) radiotherapy and surgery in the treatment of cancer, and (iii) trauma and infection. Suitable doses, formulations, and administration routes are described below.

Angiogenic polypeptides, or biologically active portions thereof, in conjunction with agonists and/or antagonists may also be used to treat Kaposi's Sarcoma. Accordingly, in a further aspect, there is provided a process for utilizing angiogenic polypeptides in conjunction with an agonist to treat Kaposi's Sarcoma. Preferably, an angiogenic polypeptide is administered to an individual for the purpose of alleviating or treating Kaposi's Sarcoma. Suitable doses, formulations, and administration routes are described below.

Antagonists of angiogenic polypeptides can be used to treat cancer by inhibiting the angiogenesis necessary to support cancer and tumor growth.

### ***Gene Therapy Methods***

Another aspect of the present invention is to gene therapy methods for treating disorders, diseases and conditions. The gene therapy methods relate to the introduction of nucleic acid (DNA, RNA and antisense DNA or RNA) sequences into an animal to achieve expression of the angiogenic polypeptide of the present invention. This method requires a polynucleotide which codes for an angiogenic polypeptide operatively linked to a promoter and any other genetic elements necessary for the expression of the polypeptide by the target tissue. Such gene therapy and delivery techniques are known in the art, see, for example, WO 90/11092, which is herein incorporated by reference.

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) comprising a promoter operably linked to a angiogenic polynucleotide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, see Belldgrun, A., *et al.*, *J. Natl. Cancer Inst.* 85: 207-216 (1993); Ferrantini, M. *et al.*, *Cancer Research* 53: 1107-1112 (1993); Ferrantini, M. *et al.*, *J. Immunology* 153: 4604-4615 (1994); Kaido, T., *et al.*, *Int. J. Cancer* 60: 221-229 (1995); Ogura, H., *et al.*, *Cancer Research* 50: 5102-5106 (1990); Santodonato, L., *et al.*, *Human Gene Therapy* 7:1-10 (1996); Santodonato, L., *et al.*, *Gene Therapy* 4:1246-1255 (1997); and Zhang, J.-F. *et al.*, *Cancer Gene Therapy* 3: 31-38 (1996)), which are herein incorporated by reference. In one embodiment, the cells which are engineered are arterial cells. The arterial cells may be reintroduced into the patient through direct injection to the artery, the tissues surrounding the artery, or through catheter injection.

As discussed in more detail below, the angiogenic polynucleotide constructs can be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, and the like). The angiogenic polynucleotide constructs may be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

In one embodiment, the angiogenic polynucleotide is delivered as a naked polynucleotide. The term "naked" polynucleotide, DNA or RNA refers to sequences that are free from any delivery vehicle that acts to assist, promote or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the angiogenic polynucleotides can also be delivered in liposome formulations and lipofectin formulations and the like can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Patent Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference.

The angiogenic polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Appropriate vectors include pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; pSVK3, pBPV, pMSG and pSVL available from Pharmacia; and pEF1/V5, pcDNA3.1, and pRc/CMV2 available from Invitrogen. Other suitable vectors will be readily apparent to the skilled artisan.

Any strong promoter known to those skilled in the art can be used for driving the expression of angiogenic DNA. Suitable promoters include adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoA1 promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs; the  $\beta$ -actin promoter; and human growth hormone promoters. The promoter also may be the native promoter for the respective gene encoding a given angiogenic protein.

Unlike other gene therapy techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

The angiogenic polynucleotide construct can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. *In vivo* muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked acid sequence injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 mg/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration.

The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked angiogenic DNA constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

The naked polynucleotides are delivered by any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous injection, topical administration, catheter infusion, and so-called "gene guns". These delivery methods are known in the art.

It has been demonstrated that naked VEGF-2 nucleic acid sequences can be administered *in vivo* which results in the successful expression of VEGF-2 polypeptide in the femoral arteries of rabbits.

The constructs may also be delivered with delivery vehicles such as viral sequences, viral particles, liposome formulations, lipofectin, precipitating agents, etc. Such methods of delivery are known in the art.

In certain embodiments, the angiogenic polynucleotide constructs are complexed in a liposome preparation. Liposomal preparations for use in the instant invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. However, cationic liposomes are particularly preferred because a tight charge complex can be formed between the cationic liposome and the polyanionic nucleic acid. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416, which is herein incorporated by reference); mRNA (Malone *et al.*, *Proc. Natl. Acad. Sci. USA* (1989) 86:6077-6081, which is herein incorporated by reference); and purified transcription factors (Debs *et al.*, *J. Biol. Chem.* (1990) 265:10189-10192, which is herein incorporated by reference), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-dioleoyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are particularly useful and are available under the trademark Lipofectin, from GIBCO BRL,

Grand Island, N.Y. (See, also, Felgner *et al.*, *Proc. Natl Acad. Sci. USA* (1987) 84:7413-7416, which is herein incorporated by reference). Other commercially available liposomes include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boehringer).

Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g. PCT Publication No. WO 90/11092 (which is herein incorporated by reference) for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes. Preparation of DOTMA liposomes is explained in the literature, see, e.g., P. Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7413-7417, which is herein incorporated by reference. Similar methods can be used to prepare liposomes from other cationic lipid materials.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, Ala.), or can be easily prepared using readily available materials. Such materials include phosphatidyl, choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphosphatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

For example, commercially dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), and dioleoylphosphatidyl ethanolamine (DOPE) can be used in various combinations to make conventional liposomes, with or without the addition of cholesterol. Thus, for example, DOPG/DOPC vesicles can be prepared by drying 50 mg each of DOPG and DOPC under a stream of nitrogen gas into a sonication vial. The sample is placed under a vacuum pump overnight and is hydrated the following day with deionized water. The sample is then sonicated for 2 hours in a capped vial, using a Heat Systems model 350 sonicator equipped with an inverted cup (bath type) probe at the maximum setting while the bath is circulated at 15EC. Alternatively, negatively charged vesicles can be prepared without sonication to produce multilamellar vesicles or by extrusion through nucleopore membranes to produce unilamellar vesicles of discrete size. Other methods are known and available to those of skill in the art.

The liposomes can comprise multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs), with SUVs being preferred. The various liposome-nucleic acid complexes are prepared using methods well known in the art. See, e.g., Straubinger *et al.*, *Methods of Immunology* (1983), 101:512-527, which is herein incorporated by reference. For example, MLVs containing nucleic acid can be prepared by depositing a thin film of phospholipid on the walls of a glass tube and subsequently hydrating with a solution of the material to be encapsulated. SUVs are prepared by extended sonication of MLVs to produce a homogeneous population of

unilamellar liposomes. The material to be entrapped is added to a suspension of preformed MLVs and then sonicated. When using liposomes containing cationic lipids, the dried lipid film is resuspended in an appropriate solution such as sterile water or an isotonic buffer solution such as 10 mM Tris/NaCl, sonicated, and then the preformed liposomes are mixed directly with the DNA. The liposome and DNA form a very stable complex due to binding of the positively charged liposomes to the cationic DNA. SUVs find use with small nucleic acid fragments. LUVs are prepared by a number of methods, well known in the art. Commonly used methods include  $\text{Ca}^{2+}$ -EDTA chelation (Papahadjopoulos *et al.*, *Biochim. Biophys. Acta* (1975) 394:483; Wilson *et al.*, *Cell* (1979) 17:77); ether injection (Deamer, D. and Bangham, A., *Biochim. Biophys. Acta* (1976) 443:629; Ostro *et al.*, *Biochem. Biophys. Res. Commun.* (1977) 76:836; Fraley *et al.*, *Proc. Natl. Acad. Sci. USA* (1979) 76:3348); detergent dialysis (Enoch, H. and Strittmatter, P., *Proc. Natl. Acad. Sci. USA* (1979) 76:145); and reverse-phase evaporation (REV) (Fraley *et al.*, *J. Biol. Chem.* (1980) 255:10431; Szoka, F. and Papahadjopoulos, D., *Proc. Natl. Acad. Sci. USA* (1978) 75:145; Schaefer-Ridder *et al.*, *Science* (1982) 215:166), which are herein incorporated by reference.

Generally, the ratio of DNA to liposomes will be from about 10:1 to about 1:10. Preferably, the ration will be from about 5:1 to about 1:5. More preferably, the ration will be about 3:1 to about 1:3. Still more preferably, the ratio will be about 1:1.

U.S. Patent No. 5,676,954 (which is herein incorporated by reference) reports on the injection of genetic material, complexed with cationic liposomes carriers, into mice. U.S. Patent Nos. 4,897,355, 4,946,787, 5,049,386, 5,459,127, 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication no. WO 94/9469 (which are herein incorporated by reference) provide cationic lipids for use in transfecting DNA into cells and mammals. U.S. Patent Nos. 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication no. WO 94/9469 (which are herein incorporated by reference) provide methods for delivering DNA-cationic lipid complexes to mammals.

In certain embodiments, cells are be engineered, *ex vivo* or *in vivo*, using a retroviral particle containing RNA which comprises a sequence encoding an angiogenic protein. Retroviruses from which the retroviral plasmid vectors may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, Rous sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, Myeloproliferative Sarcoma Virus, and mammary tumor virus.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, R-2, R-AM, PA12, T19-14X, VT-19-17-H2,

RCRE, RCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, *Human Gene Therapy* 1:5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO<sub>4</sub> precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include polynucleotide encoding an angiogenic protein. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the angiogenic protein.

In certain other embodiments, cells are engineered, *ex vivo* or *in vivo*, with angiogenic polynucleotide contained in an adenovirus vector. Adenovirus can be manipulated such that it encodes and expresses the angiogenic protein, and at the same time is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. Adenovirus expression is achieved without integration of the viral DNA into the host cell chromosome, thereby alleviating concerns about insertional mutagenesis. Furthermore, adenoviruses have been used as live enteric vaccines for many years with an excellent safety profile (Schwartz, A. R. *et al.* (1974) *Am. Rev. Respir. Dis.* 109:233-238). Finally, adenovirus mediated gene transfer has been demonstrated in a number of instances including transfer of alpha-1-antitrypsin and CFTR to the lungs of cotton rats (Rosenfeld, M. A. *et al.* (1991) *Science* 252:431-434; Rosenfeld *et al.*, (1992) *Cell* 68:143-155). Furthermore, extensive studies to attempt to establish adenovirus as a causative agent in human cancer were uniformly negative (Green, M. *et al.* (1979) *Proc. Natl. Acad. Sci. USA* 76:6606).

Suitable adenoviral vectors useful in the present invention are described, for example, in Kozarsky and Wilson, *Curr. Opin. Genet. Devel.* 3:499-503 (1993); Rosenfeld *et al.*, *Cell* 68:143-155 (1992); Engelhardt *et al.*, *Human Genet. Ther.* 4:759-769 (1993); Yang *et al.*, *Nature Genet.* 7:362-369 (1994); Wilson *et al.*, *Nature* 365:691-692 (1993); and U.S. Patent No. 5,652,224, which are herein incorporated by reference. For example, the adenovirus vector Ad2 is useful and can be grown in human 293 cells. These cells contain the E1 region of adenovirus and constitutively express Ela and Elb, which complement the defective adenoviruses by providing the products of the genes deleted from the vector. In addition to Ad2, other varieties of adenovirus (e.g., Ad3, Ad5, and Ad7) are also useful in the present invention.

Preferably, the adenoviruses used in the present invention are replication deficient. Replication deficient adenoviruses require the aid of a helper virus and/or packaging cell line to form infectious particles. The resulting virus is capable of infecting

cells and can express a polynucleotide of interest which is operably linked to a promoter, for example, the HARP promoter of the present invention, but cannot replicate in most cells. Replication deficient adenoviruses may be deleted in one or more of all or a portion of the following genes: E1a, E1b, E3, E4, E2a, or L1 through L5.

5 In certain other embodiments, the cells are engineered, *ex vivo* or *in vivo*, using an adeno-associated virus (AAV). AAVs are naturally occurring defective viruses that require helper viruses to produce infectious particles (Muzyczka, N., *Curr. Topics in Microbiol. Immunol.* 158:97 (1992)). It is also one of the few viruses that may integrate its DNA into non-dividing cells. Vectors containing as little as 300 base pairs of AAV can  
10 be packaged and can integrate, but space for exogenous DNA is limited to about 4.5 kb. Methods for producing and using such AAVs are known in the art. See, for example, U.S. Patent Nos. 5,139,941, 5,173,414, 5,354,678, 5,436,146, 5,474,935, 5,478,745, and 5,589,377.

For example, an appropriate AAV vector for use in the present invention will  
15 include all the sequences necessary for DNA replication, encapsidation, and host-cell integration. The angiogenic polynucleotide construct is inserted into the AAV vector using standard cloning methods, such as those found in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (1989). The recombinant AAV vector is then transfected into packaging cells which are infected with a helper  
20 virus, using any standard technique, including lipofection, electroporation, calcium phosphate precipitation, etc. Appropriate helper viruses include adenoviruses, cytomegaloviruses, vaccinia viruses, or herpes viruses. Once the packaging cells are transfected and infected, they will produce infectious AAV viral particles which contain the angiogenic polynucleotide construct. These viral particles are then used to transduce  
25 eukaryotic cells, either *ex vivo* or *in vivo*. The transduced cells will contain the angiogenic polynucleotide construct integrated into its genome, and will express the angiogenic protein.

Another method of gene therapy involves operably associating heterologous control regions and endogenous polynucleotide sequences (e.g. encoding an angiogenic  
30 protein) via homologous recombination (see, e.g., U.S. Patent No. 5,641,670, issued June 24, 1997; International Publication No. WO 96/29411, published September 26, 1996; International Publication No. WO 94/12650, published August 4, 1994; Koller *et al.*, *Proc. Natl. Acad. Sci. USA* 86:8932-8935 (1989); and Zijlstra *et al.*, *Nature* 342:435-438 (1989). This method involves the activation of a gene which is present in  
35 the target cells, but which is not normally expressed in the cells, or is expressed at a lower level than desired.



Polynucleotide constructs are made, using standard techniques known in the art, which contain the promoter with targeting sequences flanking the promoter. Suitable promoters are described herein. The targeting sequence is sufficiently complementary to an endogenous sequence to permit homologous recombination of the promoter-targeting sequence with the endogenous sequence. The targeting sequence will be sufficiently near the 5' end of the desired endogenous polynucleotide sequence of the angiogenic protein so the promoter will be operably linked to the endogenous sequence upon homologous recombination.

The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the amplified promoter. The amplified promoter and targeting sequences are digested and ligated together.

The promoter-targeting sequence construct is delivered to the cells, either as naked polynucleotide, or in conjunction with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, whole viruses, lipofection, precipitating agents, etc., described in more detail above. The P promoter-targeting sequence can be delivered by any method, included direct needle injection, intravenous injection, topical administration, catheter infusion, particle accelerators, etc. The methods are described in more detail below.

The promoter-targeting sequence construct is taken up by cells. Homologous recombination between the construct and the endogenous sequence takes place, such that an endogenous sequence encoding an angiogenic protein is placed under the control of the promoter. The promoter then drives the expression of the endogenous angiogenic sequence.

The polynucleotides encoding the angiogenic protein may be administered along with other polynucleotides encoding other angiogenic proteins.

Preferably, the polynucleotide encoding the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein. Typically, the signal sequence is positioned in the coding region of the polynucleotide to be expressed towards or at the 5' end of the coding region. The signal sequence may be homologous or heterologous to the polynucleotide of interest and may be homologous or heterologous to the cells to be transfected. Additionally, the signal sequence may be chemically synthesized using methods known in the art.

Any mode of administration of any of the above-described polynucleotides constructs can be used so long as the mode results in the expression of one or more molecules in an amount sufficient to provide a therapeutic effect. This includes direct needle injection, systemic injection, catheter infusion, biolistic injectors, particle  
5 accelerators (i.e., "gene guns"), gelfoam sponge depots, other commercially available depot materials, osmotic pumps (e.g., Alza minipumps), oral or suppository solid (tablet or pill) pharmaceutical formulations, and decanting or topical applications during surgery. For example, direct injection of naked calcium phosphate-precipitated plasmid into rat liver and rat spleen or a protein-coated plasmid into the portal vein has resulted in  
10 gene expression of the foreign gene in the rat livers (Kaneda *et al.*, *Science* 243:375 (1989)).

A preferred method of local administration is by direct injection. Preferably, a recombinant molecule of the present invention complexed with a delivery vehicle is administered by direct injection into or locally within the area of arteries. Administration  
15 of a composition locally within the area of arteries refers to injecting the composition centimeters and preferably, millimeters within arteries.

Another method of local administration is to contact a polynucleotide construct of the present invention in or around a surgical wound. For example, a patient can undergo surgery and the polynucleotide construct can be coated on the surface of tissue inside the  
20 wound or the construct can be injected into areas of tissue inside the wound.

Therapeutic compositions useful in systemic administration, include recombinant molecules of the present invention complexed to a targeted delivery vehicle of the present invention. Suitable delivery vehicles for use with systemic administration comprise liposomes comprising ligands for targeting the vehicle to a particular site.

Preferred methods of systemic administration, include intravenous injection, aerosol, oral and percutaneous (topical) delivery. Intravenous injections can be performed using methods standard in the art. Aerosol delivery can also be performed using methods standard in the art (see, for example, Stribling *et al.*, *Proc. Natl. Acad. Sci. USA* 189:11277-11281, 1992, which is incorporated herein by reference). Oral delivery can be  
30 performed by complexing a polynucleotide construct of the present invention to a carrier capable of withstanding degradation by digestive enzymes in the gut of an animal. Examples of such carriers, include plastic capsules or tablets, such as those known in the art. Topical delivery can be performed by mixing a polynucleotide construct of the present invention with a lipophilic reagent (e.g., DMSO) that is capable of passing into  
35 the skin.

Determining an effective amount of substance to be delivered can depend upon a number of factors including, for example, the chemical structure and biological activity of

the substance, the age and weight of the animal, the precise condition requiring treatment and its severity, and the route of administration. The frequency of treatments depends upon a number of factors, such as the amount of polynucleotide constructs administered per dose, as well as the health and history of the subject. The precise amount, number of doses, and timing of doses will be determined by the attending physician or veterinarian.

Therapeutic compositions of the present invention can be administered to any animal, preferably to mammals and birds. Preferred mammals include humans, dogs, cats, mice, rats, rabbits sheep, cattle, horses and pigs, with humans being particularly preferred.

#### ***Pharmaceutical compositions***

The angiogenic polypeptides, polynucleotides and agonists and antagonists may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide or agonist or antagonist, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the pharmaceutical compositions may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intraperitoneal, intramuscular, intratumor, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, the pharmaceutical compositions are administered in an amount of at least about 10 mg/kg body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10 mg/kg to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The angiogenic polypeptides, and agonists or antagonists which are polypeptides may also be employed in accordance with the present invention by expression of such polypeptide *in vivo*, which is often referred to as "gene therapy," described above.

Thus, for example, cells such as bone marrow cells may be engineered with a polynucleotide (DNA or RNA) encoding for the polypeptide *ex vivo*, the engineered cells are then provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding the polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo*, for example, by procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding a polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such methods should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retroviral particle, for example, an adenovirus, which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller *et al.*, *Biotechniques* 7:980-990 (1989), or any other promoter (*e.g.*, cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and b-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the

Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the b-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

5       The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, y-2, y-AM, PA12, T19-14X, VT-19-17-H2, yCRE, yCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, *Human Gene Therapy* 1:5-14 (1990), which is incorporated herein by reference in its  
10       entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and  $\text{CaPO}_4$  precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

      The producer cell line generates infectious retroviral vector particles which include  
15       the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells,  
20       hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

#### ***Diagnostic assays***

      This invention is also related to the use of the genes encoding the angiogenic  
25       proteins as part of a diagnostic assay for detecting diseases or susceptibility to diseases related to the presence of mutations in nucleic acid sequences which encode angiogenic proteins.

      Individuals carrying mutations in a gene encoding an angiogenic protein may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be  
30       obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki *et al.*, *Nature* 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding an angiogenic protein can be used to  
35       identify and analyze mutations thereof. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to

radiolabeled RNA encoding the angiogenic protein, or alternatively, radiolabeled antisense DNA sequences specific to the RNA encoding the angiogenic protein. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

5 Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different  
10 DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, *e.g.*, Myers *et al.*, *Science* 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method  
15 (*e.g.*, Cotton *et al.*, *PNAS, USA* 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (*e.g.*, Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

20 In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

The present invention also relates to a diagnostic assay for detecting altered levels of angiogenic proteins in various tissues since an over-expression of the proteins compared to normal control tissue samples may detect the presence of a disease or  
25 susceptibility to a disease, for example, abnormal cellular differentiation. Assays used to detect levels of angiogenic proteins in a sample derived from a host are well-known to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western Blot analysis, ELISA assays and "sandwich" assay. An ELISA assay (Coligan *et al.*, *Current Protocols in Immunology* 1(2), Chapter 6, (1991)) initially comprises  
30 preparing an antibody specific to the angiogenic protein antigen, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or, in this example, a horseradish peroxidase enzyme. A sample is removed from a host and incubated on a solid support, *e.g.* a polystyrene dish, that binds the  
35 proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein, such as, bovine serum albumen. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal

antibodies attach to any angiogenic protein attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to the angiogenic protein. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of angiogenic protein present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to an angiogenic protein are attached to a solid support. Polypeptides of the present invention are then labeled, for example, by radioactivity, and a sample derived from the host are passed over the solid support and the amount of label detected, for example by liquid scintillation chromatography, can be correlated to a quantity of angiogenic protein in the sample.

A "sandwich" assay is similar to an ELISA assay. In a "sandwich" assay the angiogenic protein is passed over a solid support and binds to antibody attached to a solid support. A second antibody is then bound to the angiogenic protein. A third antibody which is labeled and specific to the second antibody is then passed over the solid support and binds to the second antibody and an amount can then be quantified.

#### *Polynucleotide identification*

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphism's) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same

oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with probes from the cDNA as short as 50 or 60 base pairs. For a review of this technique, see Verma *et al.*, *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man* (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that cDNA sequence. Ultimately, complete sequencing of genes from several individuals is required to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.



### Examples

#### Example 1: In vitro Expression of Angiogenic Proteins

##### **Example 1A: Bacterial Expression and Purification of Angiogenic Proteins**

The DNA sequence encoding the angiogenic protein can be amplified using PCR oligonucleotide primers corresponding to the 5' sequences of the processed protein (minus the signal peptide sequence) and the vector sequences 3' to the gene. Additional nucleotides corresponding to the gene are added to the 5' and 3' sequences. This PCR product can be cloned into pQE60 (Qiagen, Inc. Chatsworth, CA 91311).

The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE60. pQE-60 encodes antibiotic resistance (Amp<sup>r</sup>), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-60 was then digested with NcoI and BglII. The amplified sequences are ligated into pQE-60 and are inserted in frame with the sequence encoding for the histidine tag and the ribosome binding site (RBS). The ligation mixture is then used to transform *E. coli* strain M15/rep 4 (Qiagen, Inc.) by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan<sup>r</sup>). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA is isolated and confirmed by restriction analysis. Clones containing the desired constructs are grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells are grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") is then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells are grown an extra 3 to 4 hours. Cells are then harvested by centrifugation. The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized angiogenic polypeptide is purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). The proteins are eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar

guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the proteins are dialyzed to 10 mmolar sodium phosphate.

In addition to the above expression vector, the present invention further includes  
5 an expression vector comprising phage operator and promoter elements operatively linked to a polynucleotide encoding the angiogenic polypeptide, called pHE4a. (ATCC Accession Number 209645, deposited February 25, 1998.) This vector contains: 1) a neomycinphosphotransferase gene as a selection marker, 2) an E. coli origin of replication, 3) a T5 phage promoter sequence, 4) two lac operator sequences, 5) a Shine-  
10 Delgarno sequence, and 6) the lactose operon repressor gene (lacIq). The origin of replication (oriC) is derived from pUC19 (LTI, Gaithersburg, MD). The promoter sequence and operator sequences are made synthetically.

DNA can be inserted into the pHEa by restricting the vector with NdeI and XbaI, BamHI, XhoI, or Asp718, running the restricted product on a gel, and isolating the  
15 larger fragment (the stuffer fragment should be about 310 base pairs). The DNA insert is generated according to the PCR protocol described above, using PCR primers having restriction sites for NdeI (5' primer) and XbaI, BamHI, XhoI, or Asp718 (3' primer). The PCR insert is gel purified and restricted with compatible enzymes. The insert and vector are ligated according to standard protocols.

20 The engineered vector could easily be substituted in the above protocol to express protein in a bacterial system.

#### **Example 1B: Cloning and expression of Angiogenic proteins in a baculovirus expression system**

25 The DNA sequence encoding a full length angiogenic protein is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene.

The amplified sequences are isolated from a 1% agarose gel using a commercially available kit ("GeneClean," BIO 101 Inc., La Jolla, Ca.). The fragment is then digested with the respective endonucleases and purified again on a 1% agarose gel. This fragment  
30 is designated F2.

The vector pA2 (modifications of pVL941 vector, discussed below) is used for the expression of the proteins using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No.  
35 1555). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases BamHI and XbaI. The polyadenylation

site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from E.coli is inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for the cell-mediated homologous recombination of co-transfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pA2 such as pRG1, pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., Virology, 170:31-39).

The plasmid is digested with the restriction enzymes and dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA is then isolated from a 1% agarose gel using the commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

Fragment F2 and the dephosphorylated plasmid V2 are ligated with T4 DNA ligase. E.coli DH5 alpha cells are then transformed and bacteria identified that contained the plasmid using the respective restriction enzymes. The sequence of the cloned fragment are confirmed by DNA sequencing.

5  $\mu$ g of the plasmid is co-transfected with 1.0  $\mu$ g of a commercially available linearized baculovirus ("BaculoGold baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987)).

1  $\mu$ g of BaculoGold virus DNA and 5  $\mu$ g of the plasmid is mixed in a sterile well of microtiter plates containing 50  $\mu$ l of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10  $\mu$ l Lipofectin plus 90  $\mu$ l Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to the Sf9 insect cells (ATCC CRL 1711) seeded in 35 mm tissue culture plates with 1 ml Grace's medium without serum. The plates are rocked back and forth to mix the newly added solution. The plates are then incubated for 5 hours at 27°C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plates are put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant is collected and plaque assays performed similar as described by Summers and Smith (*supra*). As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution the virus is added to the cells and blue stained plaques are picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses is then resuspended in an Eppendorf tube containing 200  $\mu$ l of Grace's medium. The agar is removed by a brief centrifugation and the supernatant  
5 containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then stored at 4°C.

Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus at a multiplicity of infection  
10 (MOI) of 2. Six hours later the medium is removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5  $\mu$ Ci of  $^{35}$ S-methionine and 5  $\mu$ Ci  $^{35}$ S cysteine (Amersham) are added. The cells are further incubated for 16 hours before they are harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

15 Microsequencing of the amino acid sequence of the amino terminus of purified protein may be used to determine the amino terminal sequence of the produced angiogenic protein.

#### 20 **Example 1C: Expression of Recombinant Angiogenic Protein in Mammalian Cells**

##### *Cloning and Expression in COS Cells*

The expression of plasmids, X-HA (the X representing an angiogenic protein of interest) derived from a vector pcDNA3/Amp (Invitrogen) containing: 1) SV40 origin of  
25 replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, an SV40 intron and polyadenylation site. DNA fragments encoding the entire precursor of the angiogenic protein and an HA tag fused in frame to the 3' end is cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag  
30 corresponds to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighten, A. Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37:767, (1984)). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

35 The plasmid construction strategy is described as follows:

The DNA sequence encoding an angiogenic polypeptide is constructed by PCR

using two primers: the 5' primer contains a BamHI site followed by 18 nucleotides of coding sequence starting from the initiation codon; the 3' primer contains complementary sequences to an XhoI site, translation stop codon, HA tag and the last 18 nucleotides of the angiogenic polypeptide coding sequence (not including the stop codon). Therefore, the PCR product contains a BamHI site, coding sequence followed by HA tag fused in frame, a translation termination stop codon next to the HA tag, and an XhoI site.

The PCR amplified DNA fragments and the vector, pcDNA3/Amp, are digested with the respective restriction enzymes and ligated. The ligation mixture is transformed into E. coli strain SURE (available from Stratagene Cloning Systems, La Jolla, CA 92037) the transformed culture is plated on ampicillin media plates and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant angiogenic polypeptide, COS cells are transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the X-HA protein is detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells are labelled for 8 hours with <sup>35</sup>S-cysteine two days post transfection. Culture media is then collected and cells are lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984)). Both cell lysate and culture media are precipitated with an HA specific monoclonal antibody. Proteins precipitated are analyzed on 15% SDS-PAGE gels.

### ***Cloning and Expression in Other Mammalian Cells***

Angiogenic polypeptide can be expressed in a mammalian cell. A typical mammalian expression vector contains a promoter element, which mediates the initiation of transcription of mRNA, a protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription is achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLV, HIV and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter).

Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2DHFR (ATCC 37146), pBC12MI (ATCC 67109), pCMVSPORT 2.0, and pCMVSPORT 3.0. Mammalian host cells that could be used include, human  
5 Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, angiogenic polypeptide can be expressed in stable cell lines containing the polynucleotide encoding the angiogenic polypeptide integrated into a chromosome. The co-transfection with a selectable marker such as DHFR, gpt,  
10 neomycin, hygromycin allows the identification and isolation of the transfected cells.

The transfected gene encoding the angiogenic polypeptide can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) marker is useful in developing cell lines that carry several hundred or even several thousand copies of the gene of interest. (See, e.g., Alt, F. W., et al., J. Biol. Chem.  
15 253:1357-1370 (1978); Hamlin, J. L. and Ma, C., Biochem. et Biophys. Acta, 1097:107-143 (1990); Page, M. J. and Sydenham, M. A., Biotechnology 9:64-68 (1991).) Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al., Biochem J. 227:277-279 (1991); Bebbington et al., Bio/Technology 10:169-175 (1992). Using these markers, the mammalian cells are grown in selective  
20 medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

Derivatives of the plasmid pSV2-DHFR (ATCC Accession No. 37146), the expression vectors pC4 (ATCC Accession No. 209646) and pC6 (ATCC Accession  
25 No.209647) contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen et al., Molecular and Cellular Biology, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart et al., Cell 41:521-530 (1985).) Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene encoding the angiogenic polypeptide. The vectors also contain the 3'  
30 intron, the polyadenylation and termination signal of the rat preproinsulin gene, and the mouse DHFR gene under control of the SV40 early promoter.

Specifically, the plasmid pC6 or pC4 is digested with appropriate restriction enzymes and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

35 The cDNA sequence encoding the full length angiogenic protein is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. If a naturally occurring signal sequence is used to produce a secreted protein, the vector

does not need a second signal peptide. Alternatively, if a naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence in an effort to secrete the protein from the cell. (See, e.g., WO 96/34891.)

The amplified fragment is then digested with the appropriate restriction enzyme and purified on a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC6 or pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene is used for transfection. Five  $\mu$ g of the expression plasmid pC6 or pC4 is cotransfected with 0.5  $\mu$ g of the plasmid pSVneo using lipofectin (Felgner et al., *supra*). The plasmid pSV2-neo contains a dominant selectable marker, the neo gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1  $\mu$ M, 2  $\mu$ M, 5  $\mu$ M, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200  $\mu$ M. Expression of angiogenic polypeptide is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

#### **Example 2: Isolation of Genomic Clones Encoding Angiogenic Polypeptides**

A human genomic P1 library (Genomic Systems, Inc.) is screened by PCR using primers selected for the cDNA sequence corresponding to SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23, respectively, according to the method of Sambrook et al.

#### **Example 3: Tissue Distribution of Angiogenic Polypeptides**

Tissue distribution of mRNA expression of an angiogenic protein is determined using protocols for Northern blot analysis, described by, among others, Sambrook et al. For example, probes specific to the gene encoding the angiogenic protein of interest are labeled with  $P^{32}$  using the rediprime™ DNA labeling system (Amersham Life Science),

according to manufacturer's instructions. After labeling, the probe is purified using CHROMA SPIN-100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for mRNA expression.

5 Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) (Clontech) are examined with the labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70 degree C overnight, and the films developed according to standard  
10 procedures.

#### **Example 4: Chromosomal Mapping of Angiogenic Proteins**

An oligonucleotide primer set is designed according to the sequence at the 5' end of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23, respectively. This primer  
15 preferably spans about 100 nucleotides. This primer set is then used in a polymerase chain reaction under the following set of conditions : 30 seconds, 95 degree C; 1 minute, 56 degree C; 1 minute, 70 degree C. This cycle is repeated 32 times followed by one 5 minute cycle at 70 degree C. Human, mouse, and hamster DNA is used as template in addition to a somatic cell hybrid panel containing individual chromosomes or  
20 chromosome fragments (Bios, Inc). The reactions is analyzed on either 8% polyacrylamide gels or 3.5 % agarose gels. Chromosome mapping is determined by the presence of an approximately 100 bp PCR fragment in the particular somatic cell hybrid.

#### **Example 5: Purification of Angiogenic Polypeptide from an Inclusion**

##### **Body**

25 The following alternative method can be used to purify angiogenic polypeptide expressed in E coli when it is present in the form of inclusion bodies. Unless otherwise specified, all of the following steps are conducted at 4-10 degree C.

Upon completion of the production phase of the E. coli fermentation, the cell  
30 culture is cooled to 4-10 degree C and the cells harvested by continuous centrifugation at 15,000 rpm (Heraeus Sepatech). On the basis of the expected yield of protein per unit weight of cell paste and the amount of purified protein required, an appropriate amount of cell paste, by weight, is suspended in a buffer solution containing 100 mM Tris, 50 mM EDTA, pH 7.4. The cells are dispersed to a homogeneous suspension using a high shear  
35 mixer.

The cells are then lysed by passing the solution through a microfluidizer (Microfluidics, Corp. or APV Gaulin, Inc.) twice at 4000-6000 psi. The homogenate is



then mixed with NaCl solution to a final concentration of 0.5 M NaCl, followed by centrifugation at 7000 xg for 15 min. The resultant pellet is washed again using 0.5M NaCl, 100 mM Tris, 50 mM EDTA, pH 7.4.

5 The resulting washed inclusion bodies are solubilized with 1.5 M guanidine hydrochloride (GuHCl) for 2-4 hours. After 7000 xg centrifugation for 15 min., the pellet is discarded and the polypeptide containing supernatant is incubated at 4 degree C overnight to allow further GuHCl extraction.

Following high speed centrifugation (30,000 xg) to remove insoluble particles, the GuHCl solubilized protein is refolded by quickly mixing the GuHCl extract with 20  
10 volumes of buffer containing 50 mM sodium, pH 4.5, 150 mM NaCl, 2 mM EDTA by vigorous stirring. The refolded diluted protein solution is kept at 4 degree C without mixing for 12 hours prior to further purification steps.

To clarify the refolded polypeptide solution, a previously prepared tangential filtration unit equipped with 0.16 um membrane filter with appropriate surface area (e.g.,  
15 Filtron), equilibrated with 40 mM sodium acetate, pH 6.0 is employed. The filtered sample is loaded onto a cation exchange resin (e.g., Poros HS-50, Perseptive Biosystems). The column is washed with 40 mM sodium acetate, pH 6.0 and eluted with 250 mM, 500 mM, 1000 mM, and 1500 mM NaCl in the same buffer, in a stepwise manner. The absorbance at 280 nm of the effluent is continuously monitored. Fractions  
20 are collected and further analyzed by SDS-PAGE.

Fractions containing the angiogenic polypeptide are then pooled and mixed with 4 volumes of water. The diluted sample is then loaded onto a previously prepared set of tandem columns of strong anion (Poros HQ-50, Perseptive Biosystems) and weak anion (Poros CM-20, Perseptive Biosystems) exchange resins. The columns are equilibrated  
25 with 40 mM sodium acetate, pH 6.0. Both columns are washed with 40 mM sodium acetate, pH 6.0, 200 mM NaCl. The CM-20 column is then eluted using a 10 column volume linear gradient ranging from 0.2 M NaCl, 50 mM sodium acetate, pH 6.0 to 1.0 M NaCl, 50 mM sodium acetate, pH 6.5. Fractions are collected under constant  $A_{280}$  monitoring of the effluent. Fractions containing the polypeptide (determined, for  
30 instance, by 16% SDS-PAGE) are then pooled.

The resultant angiogenic polypeptide should exhibit greater than 95% purity after the above refolding and purification steps. No major contaminant bands should be observed from Commassie blue stained 16% SDS-PAGE gel when 5 ug of purified protein is loaded. The purified angiogenic protein can also be tested for endotoxin/LPS  
35 contamination, and typically the LPS content is less than 0.1 ng/ml according to LAL assays.

**Example 6: Construction of N-Terminal and/or C-Terminal Deletion Mutants**

The following general approach may be used to clone a N-terminal or C-terminal deletion mutants of an angiogenic protein. Generally, two oligonucleotide primers of about 15-25 nucleotides are derived from the desired 5' and 3' positions of a polynucleotide of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23, respectively. The 5' and 3' positions of the primers are determined based on the desired polynucleotide fragment encoding the angiogenic polypeptide. An initiation and stop codon are added to the 5' and 3' primers respectively, if necessary, to express the angiogenic polypeptide fragment encoded by the polynucleotide fragment.

Additional nucleotides containing restriction sites to facilitate cloning of the polynucleotide fragment encoding the angiogenic polypeptide in a desired vector may also be added to the 5' and 3' primer sequences. The polynucleotide fragment encoding the angiogenic polypeptide is amplified from genomic DNA or a cDNA clone using the appropriate PCR oligonucleotide primers and conditions discussed herein or known in the art. The angiogenic polypeptide fragments encoded by the appropriate polynucleotide fragments of the present invention may be expressed and purified in the same general manner as the full length polypeptides, although routine modifications may be necessary due to the differences in chemical and physical properties between a particular fragment and full length polypeptide.

The amplified polynucleotide fragment and the expression vector are digested with restriction enzymes which recognize the sites in the primers. The digested polynucleotides are then ligated together. The polynucleotide fragment encoding the angiogenic polypeptide is inserted into the restricted expression vector, preferably in a manner which places the angiogenic polypeptide fragment coding region downstream from the promoter. The ligation mixture is transformed into competent E. coli cells using standard procedures and as described in the Examples herein. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

**Example 7: Protein Fusions of Angiogenic Polypeptides**

Angiogenic polypeptides are preferably fused to other proteins. These fusion proteins can be used for a variety of applications. For example, fusion of angiogenic polypeptides to His-tag, HA-tag, protein A, IgG domains, and maltose binding protein facilitates purification. (See Example 5; see also EP A 394,827; Traunecker, et al., Nature 331:84-86 (1988).) Similarly, fusion to IgG-1, IgG-3, and albumin increases the half-life time in vivo. Nuclear localization signals fused to angiogenic polypeptides can

target the protein to a specific subcellular localization, while covalent heterodimer or homodimers can increase or decrease the activity of a fusion protein. Fusion proteins can also create chimeric molecules having more than one function. Finally, fusion proteins can increase solubility and/or stability of the fused protein compared to the non-fused protein. All of the types of fusion proteins described above can be made by modifying the following protocol, which outlines the fusion of a polypeptide to an IgG molecule, or the protocol described in the Examples.

Briefly, the human Fc portion of the IgG molecule can be PCR amplified, using primers that span the 5' and 3' ends of the sequence described below. These primers also should have convenient restriction enzyme sites that will facilitate cloning into an expression vector, preferably a mammalian expression vector.

For example, if pC4 (Accession No. 209646) is used, the human Fc portion can be ligated into the BamHI cloning site. Note that the 3' BamHI site should be destroyed. Next, the vector containing the human Fc portion is re-restricted with BamHI, linearizing the vector, and polynucleotide encoding the angiogenic polypeptide, isolated by PCR, is ligated into this BamHI site. Note that the polynucleotide is cloned without a stop codon, otherwise a fusion protein will not be produced.

If the naturally occurring signal sequence is used to produce the secreted protein, pC4 does not need a second signal peptide. Alternatively, if the naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., WO 96/34891.)

Human IgG Fc region:

```
GGGATCCGGAGCCCAAATCTTCTGACAAACTCACACATGCCCACCGTGCCC
25 AGCACCTGAATTCGAGGGTGCACCGTCAGTCTTCCTCTTCCCCCAAACCCA
AGGACACCCTCATGATCTCCCGGACTCCTGAGGTACATGCGTGGTGGTGGGA
CGTAAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGT
GGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCAC
GTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGC
30 AAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAACCCCCATCGAGA
AAACCATCTCCAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCT
GCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTG
GTCAAAGGCTTCTATCCAAGCGACATCGCCGTGGAGTGGGAGAGCAATGGG
CAGCCGGAGAACAATAAGACCACGCCTCCCGTGCTGGACTCCGACGGCT
35 CTTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGG
GAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA
```

CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGAGTGCGACGGCCGCGACT  
CTAGAGGAT (SEQ ID NO:30)

### **Example 8: Production of an Antibody**

5

#### **Example 8A. Hybridoma Technology**

The antibodies of the present invention can be prepared by a variety of methods. (See, Current Protocols, Chapter 2.) As one example of such methods, cells expressing angiogenic polypeptide are administered to an animal to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of angiogenic protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

In the most preferred method, the antibodies of the present invention are monoclonal antibodies (or protein binding fragments thereof). Such monoclonal antibodies can be prepared using hybridoma technology. (Köhler et al., Nature 256:495 (1975); Köhler et al., Eur. J. Immunol. 6:511 (1976); Köhler et al., Eur. J. Immunol. 6:292 (1976); Hammerling et al., in: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981).) In general, such procedures involve immunizing an animal (preferably a mouse) with angiogenic polypeptide or, more preferably, with a secreted angiogenic polypeptide-expressing cell. Such cells may be cultured in any suitable tissue culture medium; however, it is preferable to culture cells in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (inactivated at about 56 degree C), and supplemented with about 10 g/l of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100 ug/ml of streptomycin.

The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP2O), available from the ATCC. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands et al. (Gastroenterology 80:225-232 (1981).) The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the angiogenic polypeptide.

Alternatively, additional antibodies capable of binding to angiogenic polypeptide can be produced in a two-step procedure using anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method,

protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the angiogenic protein-specific antibody can be blocked by the angiogenic protein. Such antibodies comprise anti-idiotypic antibodies to the angiogenic protein-specific antibody and can be used to immunize an animal to induce formation of further angiogenic protein-specific antibodies.

It will be appreciated that Fab and F(ab')<sub>2</sub> and other fragments of the antibodies of the present invention may be used according to the methods disclosed herein. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')<sub>2</sub> fragments). Alternatively, secreted angiogenic protein-binding fragments can be produced through the application of recombinant DNA technology or through synthetic chemistry.

For in vivo use of antibodies in humans, it may be preferable to use "humanized" chimeric monoclonal antibodies. Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric antibodies are known in the art. (See, for review, Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et al., EP 171496; Morrison et al., EP 173494; Neuberger et al., WO 8601533; Robinson et al., WO 8702671; Boulianne et al., Nature 312:643 (1984); Neuberger et al., Nature 314:268 (1985).)

#### **Example 8B. Isolation of antibody fragments directed against angiogenic polypeptide from a library of scFvs.**

Naturally occurring V-genes isolated from human PBLs are constructed into a large library of antibody fragments which contain reactivities against angiogenic polypeptides to which the donor may or may not have been exposed (see e.g., U.S. Patent 5,885,793 incorporated herein in its entirety by reference).

#### **30 Rescue of the Library.**

A library of scFvs is constructed from the RNA of human PBLs as described in WO92/01047. To rescue phage displaying antibody fragments, approximately 10<sup>9</sup> E. coli harbouring the phagemid are used to inoculate 50 ml of 2xTY containing 1% glucose and 100 ug/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture is used to inoculate 50 ml of 2xTY-AMP-GLU, 2 x 10<sup>8</sup> TU of delta gene 3 helper (M13 delta gene III, see WO92/01047) are added and the culture incubated at 37 degree C for 45 minutes without shaking and then at 37 degree C for 45

minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 liters of 2xTY containing 100 ug/ml ampicillin and 50 ug/ml kanamycin and grown overnight. Phage are prepared as described in WO92/01047.

5 M13 delta gene III is prepared as follows: M13 delta gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a greater avidity of binding to antigen. Infectious M13 delta gene III particles are made by growing the helper phage in cells harbouring a pUC19 derivative supplying the wild type gene III protein during phage morphogenesis. The culture is incubated for 1 hour at 37 degree C without shaking and then for a further hour at 37 degree C with shaking. Cells  
10 are spun down (IEC-Centra 8, 4000 revs/min for 10 min), resuspended in 300 ml 2xTY broth containing 100 ug ampicillin/ml and 25 ug kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking at 37° C. Phage particles are purified and concentrated from the culture medium by two PEG-precipitations (Sambrook et al., 1990), resuspended in 2 ml PBS and passed through a 0.45 um filter (Minisart NML; Sartorius) to give a final  
15 concentration of approximately  $10^{13}$  transducing units/ml (ampicillin-resistant clones).

#### *Panning of the Library.*

Immunotubes (Nunc) are coated overnight in PBS with 4 ml of either 100 ug/ml or 10 ug/ml of a polypeptide of the present invention. Tubes are blocked with 2% Marvel-PBS for 2 hours at 37 degree C and then washed 3 times in PBS. Approximately  
20  $10^{13}$  TU of phage is applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and  
25 over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to infect 10 ml of mid-log E. coli TG1 by incubating eluted phage with bacteria for 30 minutes at 37 degree C. The E. coli are then plated on TYE plates containing 1% glucose and 100 ug/ml ampicillin. The resulting bacterial library is then rescued with delta gene 3 helper phage as described above to  
30 prepare phage for a subsequent round of selection. This process is then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

#### *Characterization of Binders.*

35 Eluted phage from the 3rd and 4th rounds of selection are used to infect E. coli HB 2151 and soluble scFv is produced (Marks, et al., 1991) from single colonies for

assay. ELISAs are performed with microtitre plates coated with either 10 pg/ml of the polypeptide of the present invention in 50 mM bicarbonate pH 9.6. Clones positive in ELISA are further characterized by PCR fingerprinting (see e.g., WO92/01047) and then by sequencing.

5

#### **Example 9: Production Of Angiogenic Protein For Screening Assays**

The following protocol produces a supernatant containing angiogenic polypeptide to be tested. This supernatant can then be used in the Screening Assays described in Examples 14-21.

10 First, dilute Poly-D-Lysine (644 587 Boehringer-Mannheim) stock solution (1mg/ml in PBS) 1:20 in PBS (w/o calcium or magnesium 17-516F Biowhittaker) for a working solution of 50ug/ml. Add 200 ul of this solution to each well (24 well plates) and incubate at RT for 20 minutes. Be sure to distribute the solution over each well (note: a 12-channel pipetter may be used with tips on every other channel). Aspirate off  
15 the Poly-D-Lysine solution and rinse with 1ml PBS (Phosphate Buffered Saline). The PBS should remain in the well until just prior to plating the cells and plates may be poly-lysine coated in advance for up to two weeks.

Plate 293T cells (do not carry cells past P+20) at  $2 \times 10^5$  cells/well in .5ml DMEM(Dulbecco's Modified Eagle Medium)(with 4.5 G/L glucose and L-glutamine (12-  
20 604F Biowhittaker))/10% heat inactivated FBS(14-503F Biowhittaker)/1x Penstrep(17-602E Biowhittaker). Let the cells grow overnight.

The next day, mix together in a sterile solution basin: 300 ul Lipofectamine (18324-012 Gibco/BRL) and 5ml Optimem I (31985070 Gibco/BRL)/96-well plate. With a small volume multi-channel pipetter, aliquot approximately 2ug of an expression  
25 vector containing a polynucleotide insert, produced by the methods described in Examples 8-10, into an appropriately labeled 96-well round bottom plate. With a multi-channel pipetter, add 50ul of the Lipofectamine/Optimem I mixture to each well. Pipette up and down gently to mix. Incubate at RT 15-45 minutes. After about 20 minutes, use a multi-channel pipetter to add 150ul Optimem I to each well. As a control, one plate of  
30 vector DNA lacking an insert should be transfected with each set of transfections.

Preferably, the transfection should be performed by tag-teaming the following tasks. By tag-teaming, hands on time is cut in half, and the cells do not spend too much time on PBS. First, person A aspirates off the media from four 24-well plates of cells, and then person B rinses each well with .5-1ml PBS. Person A then aspirates off PBS  
35 rinse, and person B, using a 12-channel pipetter with tips on every other channel, adds the 200ul of DNA/Lipofectamine/Optimem I complex to the odd wells first, then to the even wells, to each row on the 24-well plates. Incubate at 37 degree C for 6 hours.

While cells are incubating, prepare appropriate media, either 1%BSA in DMEM with 1x penstrep, or HGS CHO-5 media (116.6 mg/L of CaCl<sub>2</sub> (anhyd); 0.00130 mg/L CuSO<sub>4</sub>·5H<sub>2</sub>O; 0.050 mg/L of Fe(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O; 0.417 mg/L of FeSO<sub>4</sub>·7H<sub>2</sub>O; 311.80 mg/L of KCl; 28.64 mg/L of MgCl<sub>2</sub>; 48.84 mg/L of MgSO<sub>4</sub>; 6995.50 mg/L of NaCl; 5 2400.0 mg/L of NaHCO<sub>3</sub>; 62.50 mg/L of NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O; 71.02 mg/L of Na<sub>2</sub>HPO<sub>4</sub>; 4320 mg/L of ZnSO<sub>4</sub>·7H<sub>2</sub>O; .002 mg/L of Arachidonic Acid ; 1.022 mg/L of Cholesterol; .070 mg/L of DL-alpha-Tocopherol-Acetate; 0.0520 mg/L of Linoleic Acid; 0.010 mg/L of Linolenic Acid; 0.010 mg/L of Myristic Acid; 0.010 mg/L of Oleic Acid; 0.010 mg/L of Palmitric Acid; 0.010 mg/L of Palmitic Acid; 100 mg/L of Pluronic F-68; 10 0.010 mg/L of Stearic Acid; 2.20 mg/L of Tween 80; 4551 mg/L of D-Glucose; 130.85 mg/ml of L- Alanine; 147.50 mg/ml of L-Arginine-HCL; 7.50 mg/ml of L-Asparagine-H<sub>2</sub>O; 6.65 mg/ml of L-Aspartic Acid; 29.56 mg/ml of L-Cystine-2HCL-H<sub>2</sub>O; 31.29 mg/ml of L-Cystine-2HCL; 7.35 mg/ml of L-Glutamic Acid; 365.0 mg/ml of L-Glutamine; 18.75 mg/ml of Glycine; 52.48 mg/ml of L-Histidine-HCL-H<sub>2</sub>O; 106.97 15 mg/ml of L-Isoleucine; 111.45 mg/ml of L-Leucine; 163.75 mg/ml of L-Lysine HCL; 32.34 mg/ml of L-Methionine; 68.48 mg/ml of L-Phenylalanine; 40.0 mg/ml of L-Proline; 26.25 mg/ml of L-Serine; 101.05 mg/ml of L-Threonine; 19.22 mg/ml of L-Tryptophan; 91.79 mg/ml of L-Tyrosine-2Na-2H<sub>2</sub>O; and 99.65 mg/ml of L-Valine; 0.0035 mg/L of Biotin; 3.24 mg/L of D-Ca Pantothenate; 11.78 mg/L of Choline Chloride; 4.65 mg/L of Folic Acid; 15.60 mg/L of i-Inositol; 3.02 mg/L of Niacinamide; 20 3.00 mg/L of Pyridoxal HCL; 0.031 mg/L of Pyridoxine HCL; 0.319 mg/L of Riboflavin; 3.17 mg/L of Thiamine HCL; 0.365 mg/L of Thymidine; 0.680 mg/L of Vitamin B<sub>12</sub>; 25 mM of HEPES Buffer; 2.39 mg/L of Na Hypoxanthine; 0.105 mg/L of Lipoic Acid; 0.081 mg/L of Sodium Putrescine-2HCL; 55.0 mg/L of Sodium Pyruvate; 25 0.0067 mg/L of Sodium Selenite; 20uM of Ethanolamine; 0.122 mg/L of Ferric Citrate; 41.70 mg/L of Methyl-B-Cyclodextrin complexed with Linoleic Acid; 33.33 mg/L of Methyl-B-Cyclodextrin complexed with Oleic Acid; 10 mg/L of Methyl-B-Cyclodextrin complexed with Retinal Acetate. Adjust osmolality to 327 mOsm) with 2mm glutamine and 1x penstrep. (BSA (81-068-3 Bayer) 100gm dissolved in 1L DMEM for a 10% BSA 30 stock solution). Filter the media and collect 50 ul for endotoxin assay in 15ml polystyrene conical.

The transfection reaction is terminated, preferably by tag-teaming, at the end of the incubation period. Person A aspirates off the transfection media, while person B adds 1.5ml appropriate media to each well. Incubate at 37 degree C for 45 or 72 hours 35 depending on the media used: 1%BSA for 45 hours or CHO-5 for 72 hours.



On day four, using a 300ul multichannel pipetter, aliquot 600ul in one 1ml deep well plate and the remaining supernatant into a 2ml deep well. The supernatants from each well can then be used in the assays described in Examples 10-12.

It is specifically understood that when activity is obtained in any of the assays described below using a supernatant, the activity originates from either the angiogenic polypeptide directly (e.g., as a secreted protein) or by angiogenic polypeptides inducing expression of other proteins, which are then secreted into the supernatant. Thus, the invention further provides a method of identifying the protein in the supernatant characterized by an activity in a particular assay.

#### **Example 10: Screening Assay Identifying Changes in Small Molecule Concentration and Membrane Permeability**

Binding of a ligand to a receptor is known to alter intracellular levels of small molecules, such as calcium, potassium, sodium, and pH, as well as alter membrane potential. These alterations can be measured in an assay to identify supernatants which bind to receptors of a particular cell. Although the following protocol describes an assay for calcium, this protocol can easily be modified to detect changes in potassium, sodium, pH, membrane potential, or any other small molecule which is detectable by a fluorescent probe.

The following assay uses Fluorometric Imaging Plate Reader ("FLIPR") to measure changes in fluorescent molecules (Molecular Probes) that bind small molecules. Clearly, any fluorescent molecule detecting a small molecule can be used instead of the calcium fluorescent molecule, fluo-3, used here.

For adherent cells, seed the cells at 10,000 -20,000 cells/well in a Co-star black 96-well plate with clear bottom. The plate is incubated in a CO<sub>2</sub> incubator for 20 hours. The adherent cells are washed two times in Biotek washer with 200 ul of HBSS (Hank's Balanced Salt Solution) leaving 100 ul of buffer after the final wash.

A stock solution of 1 mg/ml fluo-3 is made in 10% pluronic acid DMSO. To load the cells with fluo-3, 50 ul of 12 ug/ml fluo-3 is added to each well. The plate is incubated at 37 degree C in a CO<sub>2</sub> incubator for 60 min. The plate is washed four times in the Biotek washer with HBSS leaving 100 ul of buffer.

For non-adherent cells, the cells are spun down from culture media. Cells are re-suspended to  $2-5 \times 10^6$  cells/ml with HBSS in a 50-ml conical tube. 4 ul of 1 mg/ml fluo-3 solution in 10% pluronic acid DMSO is added to each ml of cell suspension. The tube is then placed in a 37 degree C water bath for 30-60 min. The cells are washed twice  
5 with HBSS, resuspended to  $1 \times 10^6$  cells/ml, and dispensed into a microplate, 100 ul/well. The plate is centrifuged at 1000 rpm for 5 min. The plate is then washed once in Denley CellWash with 200 ul, followed by an aspiration step to 100 ul final volume.

For a non-cell based assay, each well contains a fluorescent molecule, such as fluo-3. The supernatant is added to the well, and a change in fluorescence is detected.

10 To measure the fluorescence of intracellular calcium, the FLIPR is set for the following parameters: (1) System gain is 300-800 mW; (2) Exposure time is 0.4 second; (3) Camera F/stop is F/2; (4) Excitation is 488 nm; (5) Emission is 530 nm; and (6) Sample addition is 50 ul. Increased emission at 530 nm indicates an extracellular signaling event caused by the a molecule, either angiogenic polypeptide or a molecule  
15 induced by the angiogenic polypeptide, which has resulted in an increase in the intracellular  $\text{Ca}^{++}$  concentration.

#### **Example 11: Screening Assay Identifying Tyrosine Kinase Activity**

The Protein Tyrosine Kinases (PTK) represent a diverse group of transmembrane  
20 and cytoplasmic kinases. Within the Receptor Protein Tyrosine Kinase (RPTK) group are receptors for a range of mitogenic and metabolic growth factors including the PDGF, FGF, EGF, NGF, HGF and Insulin receptor subfamilies. In addition there are a large family of RPTKs for which the corresponding ligand is unknown. Ligands for RPTKs include mainly secreted small proteins, but also membrane-bound and extracellular matrix  
25 proteins.

Activation of RPTK by ligands involves ligand-mediated receptor dimerization, resulting in transphosphorylation of the receptor subunits and activation of the cytoplasmic tyrosine kinases. The cytoplasmic tyrosine kinases include receptor  
30 associated tyrosine kinases of the src-family (e.g., src, yes, lck, lyn, fyn) and non-receptor linked and cytosolic protein tyrosine kinases, such as the Jak family, members of which mediate signal transduction triggered by the cytokine superfamily of receptors (e.g., the Interleukins, Interferons, GM-CSF, and Leptin).

Because of the wide range of known factors capable of stimulating tyrosine  
35 kinase activity, identifying whether the angiogenic polypeptide or a molecule induced by the angiogenic polypeptide is capable of activating tyrosine kinase signal transduction pathways is of interest. Therefore, the following protocol is designed to identify such

molecules capable of activating the tyrosine kinase signal transduction pathways.

Seed target cells (e.g., primary keratinocytes) at a density of approximately 25,000 cells per well in a 96 well Loprodyne Silent Screen Plates purchased from Nalge Nunc (Naperville, IL). The plates are sterilized with two 30 minute rinses with 100% ethanol, rinsed with water and dried overnight. Some plates are coated for 2 hr with 100 ml of cell culture grade type I collagen (50 mg/ml), gelatin (2%) or polylysine (50 mg/ml), all of which can be purchased from Sigma Chemicals (St. Louis, MO) or 10% Matrigel purchased from Becton Dickinson (Bedford, MA), or calf serum, rinsed with PBS and stored at 4 degree C. Cell growth on these plates is assayed by seeding 5,000 cells/well in growth medium and indirect quantitation of cell number through use of alamarBlue as described by the manufacturer Alamar Biosciences, Inc. (Sacramento, CA) after 48 hr. Falcon plate covers #3071 from Becton Dickinson (Bedford, MA) are used to cover the Loprodyne Silent Screen Plates. Falcon Microtest III cell culture plates can also be used in some proliferation experiments.

To prepare extracts, A431 cells are seeded onto the nylon membranes of Loprodyne plates (20,000/200ml/well) and cultured overnight in complete medium. Cells are quiesced by incubation in serum-free basal medium for 24 hr. After 5-20 minutes treatment with EGF (60ng/ml) or 50 ul of the supernatant produced in Example 12, the medium is removed and 100 ml of extraction buffer ((20 mM HEPES pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> and a cocktail of protease inhibitors (# 1836170) obtained from Boehringer Mannheim (Indianapolis, IN) is added to each well and the plate is shaken on a rotating shaker for 5 minutes at 4°C. The plate is then placed in a vacuum transfer manifold and the extract filtered through the 0.45 mm membrane bottoms of each well using house vacuum. Extracts are collected in a 96-well catch/assay plate in the bottom of the vacuum manifold and immediately placed on ice. To obtain extracts clarified by centrifugation, the content of each well, after detergent solubilization for 5 minutes, is removed and centrifuged for 15 minutes at 4 degree C at 16,000 x g.

Test the filtered extracts for levels of tyrosine kinase activity. Although many methods of detecting tyrosine kinase activity are known, one method is described here.

Generally, the tyrosine kinase activity of a supernatant is evaluated by determining its ability to phosphorylate a tyrosine residue on a specific substrate (a biotinylated peptide). Biotinylated peptides that can be used for this purpose include PSK1 (corresponding to amino acids 6-20 of the cell division kinase cdc2-p34) and PSK2 (corresponding to amino acids 1-17 of gastrin). Both peptides are substrates for a range of tyrosine kinases and are available from Boehringer Mannheim.

The tyrosine kinase reaction is set up by adding the following components in order. First, add 10ul of 5uM Biotinylated Peptide, then 10ul ATP/Mg<sub>2</sub><sup>+</sup> (5mM ATP/50mM MgCl<sub>2</sub>), then 10ul of 5x Assay Buffer (40mM imidazole hydrochloride, pH7.3, 40 mM beta-glycerophosphate, 1mM EGTA, 100mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 0.5 mg/ml BSA), then 5ul of Sodium Vanadate(1mM), and then 5ul of water. Mix the components gently and preincubate the reaction mix at 30 degree C for 2 min. Initial the reaction by adding 10ul of the control enzyme or the filtered supernatant.

The tyrosine kinase assay reaction is then terminated by adding 10 ul of 120mM EDTA and place the reactions on ice.

Tyrosine kinase activity is determined by transferring 50 ul aliquot of reaction mixture to a microtiter plate (MTP) module and incubating at 37 degree C for 20 min. This allows the streptavidin coated 96 well plate to associate with the biotinylated peptide. Wash the MTP module with 300ul/well of PBS four times. Next add 75 ul of anti-phosphotyrosine antibody conjugated to horse radish peroxidase(anti-P-Tyr-POD(0.5u/ml)) to each well and incubate at 37 degree C for one hour. Wash the well as above.

Next add 100ul of peroxidase substrate solution (Boehringer Mannheim) and incubate at room temperature for at least 5 mins (up to 30 min). Measure the absorbance of the sample at 405 nm by using ELISA reader. The level of bound peroxidase activity is quantitated using an ELISA reader and reflects the level of tyrosine kinase activity.

#### **Example 12: Screening Assay Identifying Phosphorylation Activity**

As a potential alternative and/or compliment to the assay of protein tyrosine kinase activity described in Example 11, an assay which detects activation (phosphorylation) of major intracellular signal transduction intermediates can also be used. For example, as described below one particular assay can detect tyrosine phosphorylation of the Erk-1 and Erk-2 kinases. However, phosphorylation of other molecules, such as Raf, JNK, p38 MAP, Map kinase kinase (MEK), MEK kinase, Src, Muscle specific kinase (MuSK), IRAK, Tec, and Janus, as well as any other phosphoserine, phosphotyrosine, or phosphothreonine molecule, can be detected by substituting these molecules for Erk-1 or Erk-2 in the following assay.

Specifically, assay plates are made by coating the wells of a 96-well ELISA plate with 0.1ml of protein G (1ug/ml) for 2 hr at room temp, (RT). The plates are then rinsed with PBS and blocked with 3% BSA/PBS for 1 hr at RT. The protein G plates are then treated with 2 commercial monoclonal antibodies (100ng/well) against Erk-1 and Erk-2 (1 hr at RT) (Santa Cruz Biotechnology). (To detect other molecules, this step can easily be

modified by substituting a monoclonal antibody detecting any of the above described molecules.) After 3-5 rinses with PBS, the plates are stored at 4 degree C until use.

A431 cells are seeded at 20,000/well in a 96-well Loprodyn filterplate and cultured overnight in growth medium. The cells are then starved for 48 hr in basal medium (DMEM) and then treated with EGF (6ng/well) or 50 ul of the supernatants  
5 obtained in Example 12 for 5-20 minutes. The cells are then solubilized and extracts filtered directly into the assay plate.

After incubation with the extract for 1 hr at RT, the wells are again rinsed. As a positive control, a commercial preparation of MAP kinase (10ng/well) is used in place of  
10 A431 extract. Plates are then treated with a commercial polyclonal (rabbit) antibody (1ug/ml) which specifically recognizes the phosphorylated epitope of the Erk-1 and Erk-2 kinases (1 hr at RT). This antibody is biotinylated by standard procedures. The bound polyclonal antibody is then quantitated by successive incubations with Europium-streptavidin and Europium fluorescence enhancing reagent in the Wallac DELFIA  
15 instrument (time-resolved fluorescence). An increased fluorescent signal over background indicates a phosphorylation by the angiogenic polypeptide or a molecule induced by the angiogenic polypeptide.

**Example 13: Method of Determining Alterations in the Gene Encoding the**  
20 **Angiogenic Polypeptide**

RNA isolated from entire families or individual patients presenting with a phenotype of interest (such as a disease) is isolated. cDNA is then generated from these RNA samples using protocols known in the art. (See, Sambrook.) The cDNA is then used as a template for PCR, employing primers surrounding regions of interest in the  
25 nucleotide sequence encoding the respective angiogenic polypeptide. Suggested PCR conditions consist of 35 cycles at 95 degree C for 30 seconds; 60-120 seconds at 52-58 degree C; and 60-120 seconds at 70 degree C, using buffer solutions described in Sidransky, D., et al., Science 252:706 (1991).

PCR products are then sequenced using primers labeled at their 5' end with T4  
30 polynucleotide kinase, employing SequiTherm Polymerase. (Epicentre Technologies). The intron-exon borders of selected exons of the gene encoding the angiogenic polypeptide is also determined and genomic PCR products analyzed to confirm the results. PCR products harboring suspected mutations in the gene encoding the angiogenic polypeptide are then cloned and sequenced to validate the results of the direct  
35 sequencing.

PCR products of the gene encoding the angiogenic polypeptide are cloned into T-tailed vectors as described in Holton, T.A. and Graham, M.W., Nucleic Acids Research,

19:1156 (1991) and sequenced with T7 polymerase (United States Biochemical). Affected individuals are identified by mutations in the gene encoding the angiogenic polypeptide not present in unaffected individuals.

Genomic rearrangements are also observed as a method of determining alterations in the gene encoding the angiogenic polypeptide. Genomic clones isolated according to Example 2 are nick-translated with digoxigenin deoxy-uridine 5'-triphosphate (Boehringer Mannheim), and FISH performed as described in Johnson, Cg. et al., Methods Cell Biol. 35:73-99 (1991). Hybridization with the labeled probe is carried out using a vast excess of human cot-1 DNA for specific hybridization to the genomic locus encoding the angiogenic polypeptide.

Chromosomes are counterstained with 4,6-diamino-2-phenylidole and propidium iodide, producing a combination of C- and R-bands. Aligned images for precise mapping are obtained using a triple-band filter set (Chroma Technology, Brattleboro, VT) in combination with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) and variable excitation wavelength filters. (Johnson, Cv. et al., Genet. Anal. Tech. Appl., 8:75 (1991).) Image collection, analysis and chromosomal fractional length measurements are performed using the ISee Graphical Program System. (Inovision Corporation, Durham, NC.) Chromosome alterations of the genomic region of the gene encoding the angiogenic polypeptide (hybridized by the probe) are identified as insertions, deletions, and translocations. These alterations are used as a diagnostic marker for an associated disease.

#### **Example 14: Method of Detecting Abnormal Levels of Angiogenic Polypeptide in a Biological Sample**

Angiogenic polypeptides can be detected in a biological sample, and if an increased or decreased level of the angiogenic polypeptide is detected, this polypeptide is a marker for a particular phenotype. Methods of detection are numerous, and thus, it is understood that one skilled in the art can modify the following assay to fit their particular needs.

For example, antibody-sandwich ELISAs are used to detect the angiogenic polypeptide in a sample, preferably a biological sample. Wells of a microtiter plate are coated with specific antibodies to the angiogenic polypeptide, at a final concentration of 0.2 to 10 ug/ml. The antibodies are either monoclonal or polyclonal and are produced by the method described in Example 8. The wells are blocked so that non-specific binding of the angiogenic polypeptide to the well is reduced.

The coated wells are then incubated for > 2 hours at RT with a sample containing the angiogenic polypeptide. Preferably, serial dilutions of the sample should be used to

validate results. The plates are then washed three times with deionized or distilled water to remove unbounded angiogenic polypeptide.

Next, 50 ul of specific antibody-alkaline phosphatase conjugate, at a concentration of 25-400 ng, is added and incubated for 2 hours at room temperature. The plates are again washed three times with deionized or distilled water to remove unbounded conjugate.

Add 75 ul of 4-methylumbelliferyl phosphate (MUP) or p-nitrophenyl phosphate (NPP) substrate solution to each well and incubate 1 hour at room temperature. Measure the reaction by a microtiter plate reader. Prepare a standard curve, using serial dilutions of a control sample, and plot angiogenic polypeptide concentration on the X-axis (log scale) and fluorescence or absorbance of the Y-axis (linear scale). Interpolate the concentration of the angiogenic polypeptide in the sample using the standard curve.

#### **Example 15: Formulation**

The invention also provides methods of treatment and/or prevention of diseases, disorders, and/or conditions (such as, for example, any one or more of the diseases, disorders, and/or conditions disclosed herein) by administration to a subject of an effective amount of a Therapeutic. By therapeutic is meant a polynucleotides or polypeptides of the invention (including fragments and variants), agonists or antagonists thereof, and/or antibodies thereto, in combination with a pharmaceutically acceptable carrier type (e.g., a sterile carrier).

The Therapeutic will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the individual patient (especially the side effects of treatment with the Therapeutic alone), the site of delivery, the method of administration, the scheduling of administration, and other factors known to practitioners. The "effective amount" for purposes herein is thus determined by such considerations.

As a general proposition, the total pharmaceutically effective amount of the Therapeutic administered parenterally per dose will be in the range of about 1ug/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the Therapeutic is typically administered at a dose rate of about 1 ug/kg/hour to about 50 ug/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed. The length of treatment needed to observe changes and the

interval following treatment for responses to occur appears to vary depending on the desired effect.

Therapeutics can be administered orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), buccally, or as an oral or nasal spray. "Pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Therapeutics of the invention are suitably administered by sustained-release systems. Suitable examples of sustained-release Therapeutics are administered orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), buccally, or as an oral or nasal spray. "Pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Therapeutics of the invention are also suitably administered by sustained-release systems. Suitable examples of sustained-release Therapeutics include suitable polymeric materials (such as, for example, semi-permeable polymer matrices in the form of shaped articles, e.g., films, or microcapsules), suitable hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, and sparingly soluble derivatives (such as, for example, a sparingly soluble salt).

Sustained-release matrices include polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (Sidman, U. et al., *Biopolymers* 22:547-556 (1983)), poly (2-hydroxyethyl methacrylate) (R. Langer et al., *J. Biomed. Mater. Res.* 15:167-277 (1981), and R. Langer, *Chem. Tech.* 12:98-105 (1982)), ethylene vinyl acetate (R. Langer et al.) or poly-D- (-)-3-hydroxybutyric acid (EP 133,988).

Sustained-release therapeutics also include liposomally entrapped Therapeutics of the invention (*see generally*, Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 317-327 and 353-365 (1989)). Liposomes containing Therapeutic are prepared by methods known per se: DE 3,218,121; Epstein et al., *Proc. Natl. Acad. Sci. USA* 82:3688-3692 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA* 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641;



Japanese Pat. Appl. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal Therapeutic.

5 In yet an additional embodiment, the Therapeutics of the invention are delivered by way of a pump (*see* Langer, *supra*; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)).

10 Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

For parenteral administration, in one embodiment, the Therapeutic is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is  
15 compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to the Therapeutic.

Generally, the formulations are prepared by contacting the Therapeutic uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if  
20 necessary, the product is shaped into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

25 The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine  
30 or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, manose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium;  
35 and/or nonionic surfactants such as polysorbates, poloxamers, or PEG.

The Therapeutic is typically formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 3 to 8. It will be

understood that the use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of polypeptide salts.

Therapeutics used for therapeutic administration can be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron  
5 membranes). Therapeutic polypeptide compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Therapeutics polypeptides ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a  
10 lyophilized formulation for reconstitution. As an example of a lyophilized formulation, 10-ml vials are filled with 5 ml of sterile-filtered 1% (w/v) aqueous Therapeutics solution, and the resulting mixture is lyophilized. The infusion solution is prepared by reconstituting the lyophilized Therapeutics using bacteriostatic Water-for-Injection.

The invention also provides a pharmaceutical pack or kit comprising one or more  
15 containers filled with one or more of the ingredients of the Therapeutics of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the Therapeutic may be employed in  
20 conjunction with other therapeutic compounds.

The Therapeutics of the invention may be administered alone or in combination with adjuvants. Adjuvants that may be administered with the Therapeutics of the invention include, but are not limited to, alum, alum plus deoxycholate (ImmunoAg), MTP-PE (Biocine Corp.), QS21 (Genentech, Inc.), BCG, and MPL. In a specific  
25 embodiment, Therapeutics of the invention are administered in combination with alum. In another specific embodiment, Therapeutics of the invention are administered in combination with QS-21. Further adjuvants that may be administered with the Therapeutics of the invention include, but are not limited to, Monophosphoryl lipid immunomodulator, AdjuVax 100a, QS-21, QS-18, CRL1005, Aluminum salts, MF-59,  
30 and Virosomal adjuvant technology. Vaccines that may be administered with the Therapeutics of the invention include, but are not limited to, vaccines directed toward protection against MMR (measles, mumps, rubella), polio, varicella, tetanus/diphtheria, hepatitis A, hepatitis B, haemophilus influenzae B, whooping cough, pneumonia, influenza, Lyme's Disease, rotavirus, cholera, yellow fever, Japanese encephalitis,  
35 poliomyelitis, rabies, typhoid fever, and pertussis. Combinations may be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents

are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

The Therapeutics of the invention may be administered alone or in combination with other therapeutic agents. Therapeutic agents that may be administered in combination with the compositions of the invention, include but not limited to, other members of the TNF family, chemotherapeutic agents, antibiotics, steroidal and non-steroidal anti-inflammatories, conventional immunotherapeutic agents, cytokines and/or growth factors. Combinations may be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

In one embodiment, the Therapeutics of the invention are administered in combination with other members of the TNF family. TNF, TNF-related or TNF-like molecules that may be administered with the compositions of the invention include, but are not limited to, soluble forms of TNF-alpha, lymphotoxin-alpha (LT-alpha, also known as TNF-beta), LT-beta (found in complex heterotrimer LT-alpha2-beta), OPGL, FasL, CD27L, CD30L, CD40L, 4-1BBL, DcR3, OX40L, TNF-gamma (International Publication No. WO 96/14328), AIM-I (International Publication No. WO 97/33899), endokine-alpha (International Publication No. WO 98/07880), TR6 (International Publication No. WO 98/30694), OPG, and neutrokin-alpha (International Publication No. WO 98/18921, OX40, and nerve growth factor (NGF), and soluble forms of Fas, CD30, CD27, CD40 and 4-1BB, TR2 (International Publication No. WO 96/34095), DR3 (International Publication No. WO 97/33904), DR4 (International Publication No. WO 98/32856), TR5 (International Publication No. WO 98/30693), TR6 (International Publication No. WO 98/30694), TR7 (International Publication No. WO 98/41629), TRANK, TR9 (International Publication No. WO 98/56892), TR10 (International Publication No. WO 98/54202), 312C2 (International Publication No. WO 98/06842), and TR12, and soluble forms CD154, CD70, and CD153.

In certain embodiments, Therapeutics of the invention are administered in combination with antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors. Nucleoside

reverse transcriptase inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, RETROVIR™

(zidovudine/AZT), VIDEX™ (didanosine/ddI), HIVID™ (zalcitabine/ddC), ZERIT™ (stavudine/d4T), EPIVIR™ (lamivudine/3TC), and COMBIVIR™

5 (zidovudine/lamivudine). Non-nucleoside reverse transcriptase inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, VIRAMUNE™ (nevirapine), RESCRIPTOR™ (delavirdine), and SUSTIVA™ (efavirenz). Protease inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, CRIXIVAN™ (indinavir),

10 NORVIR™ (ritonavir), INVIRASE™ (saquinavir), and VIRACEPT™ (nelfinavir). In a specific embodiment, antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors may be used in any combination with Therapeutics of the invention to treat AIDS and/or to prevent or treat HIV infection.

15 In other embodiments, Therapeutics of the invention may be administered in combination with anti-opportunistic infection agents. Anti-opportunistic agents that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, TRIMETHOPRIM-SULFAMETHOXAZOLE™, DAPSONE™,

PENTAMIDINE™, ATOVAQUONE™, ISONIAZID™, RIFAMPIN™, 20 PYRAZINAMIDE™, ETHAMBUTOL™, RIFABUTIN™, CLARITHROMYCIN™, AZITHROMYCIN™, GANCICLOVIR™, FOSCARNET™, CIDOFOVIR™, FLUCONAZOLE™, ITRACONAZOLE™, KETOCONAZOLE™, ACYCLOVIR™, FAMCICOLVIR™, PYRIMETHAMINE™, LEUCOVORIN™, NEUPOGEN™

(filgrastim/G-CSF), and LEUKINE™ (sargramostim/GM-CSF). In a specific 25 embodiment, Therapeutics of the invention are used in any combination with TRIMETHOPRIM-SULFAMETHOXAZOLE™, DAPSONE™, PENTAMIDINE™, and/or ATOVAQUONE™ to prophylactically treat and/or prevent an opportunistic *Pneumocystis carinii* pneumonia infection. In another specific embodiment, Therapeutics of the invention are used in any combination with ISONIAZID™, RIFAMPIN™,

PYRAZINAMIDE™, and/or ETHAMBUTOL™ to prophylactically treat and/or prevent an opportunistic *Mycobacterium avium* complex infection. In another specific embodiment, Therapeutics of the invention are used in any combination with RIFABUTIN™, CLARITHROMYCIN™, and/or AZITHROMYCIN™ to

5 prophylactically treat and/or prevent an opportunistic *Mycobacterium tuberculosis* infection. In another specific embodiment, Therapeutics of the invention are used in any combination with GANCICLOVIR™, FOSCARNET™, and/or CIDOFOVIR™ to prophylactically treat and/or prevent an opportunistic cytomegalovirus infection. In another specific embodiment, Therapeutics of the invention are used in any combination  
10 with FLUCONAZOLE™, ITRACONAZOLE™, and/or KETOCONAZOLE™ to prophylactically treat and/or prevent an opportunistic fungal infection. In another specific embodiment, Therapeutics of the invention are used in any combination with ACYCLOVIR™ and/or FAMCICOLVIR™ to prophylactically treat and/or prevent an opportunistic herpes simplex virus type I and/or type II infection. In another specific  
15 embodiment, Therapeutics of the invention are used in any combination with PYRIMETHAMINE™ and/or LEUCOVORIN™ to prophylactically treat and/or prevent an opportunistic *Toxoplasma gondii* infection. In another specific embodiment, Therapeutics of the invention are used in any combination with LEUCOVORIN™ and/or NEUPOGEN™ to prophylactically treat and/or prevent an opportunistic bacterial  
20 infection.

In a further embodiment, the Therapeutics of the invention are administered in combination with an antiviral agent. Antiviral agents that may be administered with the Therapeutics of the invention include, but are not limited to, acyclovir, ribavirin, amantadine, and remantidine.

25 In a further embodiment, the Therapeutics of the invention are administered in combination with an antibiotic agent. Antibiotic agents that may be administered with the Therapeutics of the invention include, but are not limited to, amoxicillin, beta-lactamases, aminoglycosides, beta-lactam (glycopeptide), beta-lactamases, Clindamycin, chloramphenicol, cephalosporins, ciprofloxacin, ciprofloxacin, erythromycin,  
30 fluoroquinolones, macrolides, metronidazole, penicillins, quinolones, rifampin, streptomycin, sulfonamide, tetracyclines, trimethoprim, trimethoprim-sulfamthoxazole, and vancomycin.

Conventional nonspecific immunosuppressive agents, that may be administered in combination with the compositions of the invention include, but are not limited to, steroids, cyclosporine, cyclosporine analogs, cyclophosphamide methylprednisone, prednisone, azathioprine, FK-506, 15-deoxyspergualin, and other immunosuppressive agents that act by suppressing the function of responding T cells.

In specific embodiments, Therapeutics of the invention are administered in combination with immunosuppressants. Immunosuppressants preparations that may be administered with the Therapeutics of the invention include, but are not limited to, ORTHOCLONE™ (OKT3), SANDIMMUNE™/NEORAL™/SANGDYA™

(cyclosporin), PROGRAF™ (tacrolimus), CELLCEPT™ (mycophenolate), Azathioprine, glucocorticosteroids, and RAPAMUNE™ (sirolimus). In a specific embodiment, immunosuppressants may be used to prevent rejection of organ or bone marrow transplantation.

In an additional embodiment, Therapeutics of the invention are administered alone or in combination with one or more intravenous immune globulin preparations. Intravenous immune globulin preparations that may be administered with the Therapeutics of the invention include, but not limited to, GAMMAR™, IVEEGAM™, SANDOGLOBULIN™, GAMMAGARD S/D™, and GAMIMUNE™. In a specific embodiment, Therapeutics of the invention are administered in combination with intravenous immune globulin preparations in transplantation therapy (e.g., bone marrow transplant).

In a further embodiment, the compositions of the invention are administered in combination with an antibiotic agent. Antibiotic agents that may be administered with the compositions of the invention include, but are not limited to, tetracycline, metronidazole, amoxicillin, beta-lactamases, aminoglycosides, macrolides, quinolones, fluoroquinolones, cephalosporins, erythromycin, ciprofloxacin, and streptomycin.

In an additional embodiment, the compositions of the invention are administered alone or in combination with an anti-inflammatory agent. Anti-inflammatory agents that may be administered with the compositions of the invention include, but are not limited to, glucocorticoids and the nonsteroidal anti-inflammatories, aminoarylcarboxylic acid derivatives, arylacetic acid derivatives, arylbutyric acid derivatives, arylcarboxylic acids, arylpropionic acid derivatives, pyrazoles, pyrazolones, salicylic acid derivatives, thiazinecarboxamides, e-acetamidocaproic acid, S-adenosylmethionine, 3-amino-4-hydroxybutyric acid, amixetrine, bendazac, benzydamine, bucolome, difenpiramide,

ditazol, emorfazone, guaiazulene, nabumetone, nimesulide, orgotein, oxaceprol, paranyline, perisoxal, pifoxime, proquazone, proxazole, and tenidap.

In another embodiment, compositions of the invention are administered in combination with a chemotherapeutic agent. Chemotherapeutic agents that may be administered with the compositions of the invention include, but are not limited to, antibiotic derivatives (e.g., doxorubicin, bleomycin, daunorubicin, and dactinomycin); antiestrogens (e.g., tamoxifen); antimetabolites (e.g., fluorouracil, 5-FU, methotrexate, floxuridine, interferon alpha-2b, glutamic acid, plicamycin, mercaptopurine, and 6-thioguanine); cytotoxic agents (e.g., carmustine, BCNU, lomustine, CCNU, cytosine arabinoside, cyclophosphamide, estramustine, hydroxyurea, procarbazine, mitomycin, busulfan, cis-platin, and vincristine sulfate); hormones (e.g., medroxyprogesterone, estramustine phosphate sodium, ethinyl estradiol, estradiol, megestrol acetate, methyltestosterone, diethylstilbestrol diphosphate, chlorotrianisene, and testolactone); nitrogen mustard derivatives (e.g., mephallen, chorambucil, mechlorethamine (nitrogen mustard) and thiotepe); steroids and combinations (e.g., bethamethasone sodium phosphate); and others (e.g., dicarbazine, asparaginase, mitotane, vincristine sulfate, vinblastine sulfate, and etoposide).

In a specific embodiment, Therapeutics of the invention are administered in combination with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or any combination of the components of CHOP. In another embodiment, Therapeutics of the invention are administered in combination with Rituximab. In a further embodiment, Therapeutics of the invention are administered with Rituxmab and CHOP, or Rituxmab and any combination of the components of CHOP.

In an additional embodiment, the compositions of the invention are administered in combination with cytokines. Cytokines that may be administered with the compositions of the invention include, but are not limited to, IL2, IL3, IL4, IL5, IL6, IL7, IL10, IL12, IL13, IL15, anti-CD40, CD40L, IFN-gamma and TNF-alpha. In another embodiment, Therapeutics of the invention may be administered with any interleukin, including, but not limited to, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, and IL-21.

In an additional embodiment, the compositions of the invention are administered in combination with angiogenic proteins. Angiogenic proteins that may be administered with the compositions of the invention include, but are not limited to, Glioma Derived Growth Factor (GDGF), as disclosed in European Patent Number EP-399816; Platelet Derived Growth Factor-A (PDGF-A), as disclosed in European Patent Number EP-682110; Platelet Derived Growth Factor-B (PDGF-B), as disclosed in European Patent

Number EP-282317; Placental Growth Factor (PlGF), as disclosed in International Publication Number WO 92/06194; Placental Growth Factor-2 (PlGF-2), as disclosed in Hauser et al., Growth Factors, 4:259-268 (1993); Vascular Endothelial Growth Factor (VEGF), as disclosed in International Publication Number WO 90/13649; Vascular Endothelial Growth Factor-A (VEGF-A), as disclosed in European Patent Number EP-506477; Vascular Endothelial Growth Factor-2 (VEGF-2), as disclosed in International Publication Number WO 96/39515; Vascular Endothelial Growth Factor B-186 (VEGF-B186), as disclosed in International Publication Number WO 96/26736; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/02543; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/07832; and Vascular Endothelial Growth Factor-E (VEGF-E), as disclosed in German Patent Number DE19639601. The above mentioned references are incorporated herein by reference herein.

In an additional embodiment, the compositions of the invention are administered in combination with Fibroblast Growth Factors. Fibroblast Growth Factors that may be administered with the compositions of the invention include, but are not limited to, FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8, FGF-9, FGF-10, FGF-11, FGF-12, FGF-13, FGF-14, and FGF-15.

In additional embodiments, the compositions of the invention are administered in combination with other therapeutic or prophylactic regimens, such as, for example, radiation therapy.

#### **Example 16: Method of Treating Decreased Levels of Angiogenic Protein**

The present invention also relates to a method for treating an individual in need of an increased level of angiogenic polypeptide activity in the body comprising administering to such an individual a composition comprising a therapeutically effective amount of angiogenic polypeptide or an agonist thereof.

Moreover, it will be appreciated that conditions caused by a decrease in the standard or normal expression level of angiogenic polypeptide in an individual can be treated by administering angiogenic polypeptide, preferably in the secreted form. Thus, the invention also provides a method of treatment of an individual in need of an increased level of angiogenic polypeptide comprising administering to such an individual a pharmaceutical composition comprising an amount of angiogenic polypeptide to increase the activity level of angiogenic polypeptide in such an individual.

For example, a patient with decreased levels of angiogenic polypeptide receives a daily dose 0.1-100 ug/kg of the polypeptide for six consecutive days. Preferably, the



polypeptide is in the secreted form. The exact details of the dosing scheme, based on administration and formulation, are provided in Example 15.

**Example 17: Method of Treating Increased Levels of Angiogenic Protein**

5

The present invention relates to a method for treating an individual in need of a decreased level of angiogenic polypeptide activity in the body comprising, administering to such an individual a composition comprising a therapeutically effective amount of angiogenic polypeptide antagonist. Preferred antagonists for use in the present invention are angiogenic polypeptide-specific antibodies.

10

Antisense technology is used to inhibit production of angiogenic polypeptides. This technology is one example of a method of decreasing levels of angiogenic polypeptide, preferably a secreted form, due to a variety of etiologies, such as cancer.

15

For example, a patient diagnosed with abnormally increased levels of angiogenic polypeptide is administered intravenously antisense polynucleotides at 0.5, 1.0, 1.5, 2.0 and 3.0 mg/kg day for 21 days. This treatment is repeated after a 7-day rest period if the treatment is well tolerated. The formulation of the antisense polynucleotide is provided in Example 15.

20

**Example 18: Method of Treatment Using Gene Therapy - Ex Vivo**

25

One method of gene therapy transplants fibroblasts, which are capable of expressing angiogenic polypeptides, onto a patient. Generally, fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin) is added. The flasks are then incubated at 37 degree C for approximately one week.

30

At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

35

pMV-7 (Kirschmeier, P.T. et al., DNA, 7:219-25 (1988)), flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding angiogenic polypeptides can be amplified using PCR primers which correspond to the 5' and 3' end sequences respectively as set forth in Example 1. Preferably, the 5' primer contains an EcoRI site and the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is then used to transform bacteria HB101, which are then plated onto agar containing kanamycin for the purpose of confirming that the vector contains properly inserted gene encoding the angiogenic polypeptide.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene encoding the angiogenic polypeptide is then added to the media and the packaging cells transduced with the vector. The packaging cells now produce infectious viral particles containing the gene encoding the angiogenic polypeptide (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his. Once the fibroblasts have been efficiently infected, the fibroblasts are analyzed to determine whether the angiogenic protein is produced. The engineered fibroblasts are then transplanted onto the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads.

#### **Example 19: Gene Therapy Using Endogenous Gene Encoding the Angiogenic Protein**

Another method of gene therapy according to the present invention involves operably associating the endogenous sequence encoding the angiogenic protein with a promoter via homologous recombination as described, for example, in U.S. Patent No. 5,641,670, issued June 24, 1997; International Publication No. WO 96/29411, published September 26, 1996; International Publication No. WO 94/12650, published August 4, 1994; Koller et al., Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); and

Zijlstra et al., Nature 342:435-438 (1989). This method involves the activation of a gene which is present in the target cells, but which is not expressed in the cells, or is expressed at a lower level than desired.

Polynucleotide constructs are made which contain a promoter and targeting sequences, which are homologous to the 5' non-coding sequence of the endogenous gene encoding the angiogenic polypeptide, flanking the promoter. The targeting sequence will be sufficiently near the 5' end of the gene so that the promoter will be operably linked to the endogenous sequence upon homologous recombination. The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the amplified promoter. The amplified promoter and the amplified targeting sequences are digested with the appropriate restriction enzymes and subsequently treated with calf intestinal phosphatase. The digested promoter and digested targeting sequences are added together in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The construct is size fractionated on an agarose gel then purified by phenol extraction and ethanol precipitation.

In this Example, the polynucleotide constructs are administered as naked polynucleotides via electroporation. However, the polynucleotide constructs may also be administered with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, precipitating agents, etc. Such methods of delivery are known in the art.

Once the cells are transfected, homologous recombination will take place which results in the promoter being operably linked to the endogenous gene sequence. This results in the expression of an angiogenic polypeptide in the cell. Expression may be detected by immunological staining, or any other method known in the art.

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in DMEM + 10% fetal calf serum. Exponentially growing or early stationary phase fibroblasts are trypsinized and rinsed from the plastic surface with nutrient medium. An aliquot of the cell suspension is removed for counting, and the remaining cells are subjected to centrifugation. The supernatant is aspirated and the pellet is resuspended in 5 ml of electroporation buffer (20 mM HEPES pH 7.3, 137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub> HPO<sub>4</sub>, 6 mM dextrose). The cells are recentrifuged, the supernatant aspirated, and the cells resuspended in electroporation buffer containing 1 mg/ml acetylated bovine serum albumin. The final cell suspension contains

approximately  $3 \times 10^6$  cells/ml. Electroporation should be performed immediately following resuspension.

Plasmid DNA is prepared according to standard techniques. For example, to construct a plasmid for targeting to the locus where the gene encoding the angiogenic protein is located, plasmid pUC18 (MBI Fermentas, Amherst, NY) is digested with HindIII. The CMV promoter is amplified by PCR with an XbaI site on the 5' end and a BamHI site on the 3' end. Two angiogenic protein non-coding sequences are amplified via PCR: one angiogenic protein non-coding sequence (angiogenic protein fragment 1) is amplified with a HindIII site at the 5' end and an Xba site at the 3' end; the other angiogenic protein non-coding sequence (angiogenic protein fragment 2) is amplified with a BamHI site at the 5' end and a HindIII site at the 3' end. The CMV promoter and angiogenic protein fragments are digested with the appropriate enzymes (CMV promoter - XbaI and BamHI; angiogenic protein fragment 1 - XbaI; angiogenic protein fragment 2 - BamHI) and ligated together. The resulting ligation product is digested with HindIII, and ligated with the HindIII-digested pUC18 plasmid.

Plasmid DNA is added to a sterile cuvette with a 0.4 cm electrode gap (Bio-Rad). The final DNA concentration is generally at least  $120 \mu\text{g/ml}$ . 0.5 ml of the cell suspension (containing approximately  $1.5 \times 10^6$  cells) is then added to the cuvette, and the cell suspension and DNA solutions are gently mixed. Electroporation is performed with a Gene-Pulser apparatus (Bio-Rad). Capacitance and voltage are set at  $960 \mu\text{F}$  and 250-300 V, respectively. As voltage increases, cell survival decreases, but the percentage of surviving cells that stably incorporate the introduced DNA into their genome increases dramatically. Given these parameters, a pulse time of approximately 14-20 mSec should be observed.

Electroporated cells are maintained at room temperature for approximately 5 min, and the contents of the cuvette are then gently removed with a sterile transfer pipette. The cells are added directly to 10 ml of prewarmed nutrient media (DMEM with 15% calf serum) in a 10 cm dish and incubated at 37 degree C. The following day, the media is aspirated and replaced with 10 ml of fresh media and incubated for a further 16-24 hours.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product. The fibroblasts can then be introduced into a patient as described above.

#### **Example 20: Method of Treatment Using Gene Therapy - In Vivo**

Another aspect of the present invention is using in vivo gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the

introduction of naked nucleic acid (DNA, RNA, and antisense DNA or RNA) sequences encoding angiogenic polypeptides into an animal to increase or decrease the expression of the angiogenic polypeptide. The polynucleotide encoding the angiogenic polypeptides may be operatively linked to a promoter or any other genetic elements necessary for the expression of the angiogenic polypeptide by the target tissue. Such gene therapy and delivery techniques and methods are known in the art, see, for example, WO90/11092, WO98/11779; U.S. Patent NO. 5693622, 5705151, 5580859; Tabata H. et al. (1997) Cardiovasc. Res. 35(3):470-479, Chao J et al. (1997) Pharmacol. Res. 35(6):517-522, Wolff J.A. (1997) Neuromuscul. Disord. 7(5):314-318, Schwartz B. et al. (1996) Gene Ther. 3(5):405-411, Tsurumi Y. et al. (1996) Circulation 94(12):3281-3290 (incorporated herein by reference).

The polynucleotide constructs containing the gene encoding the angiogenic polypeptides may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). Further, the polynucleotide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

The term "naked" polynucleotide, DNA or RNA, refers to sequences that are free from any delivery vehicle that acts to assist, promote, or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the polynucleotides encoding the angiogenic polypeptides may also be delivered in liposome formulations (such as those taught in Felgner P.L. et al. (1995) Ann. NY Acad. Sci. 772:126-139 and Abdallah B. et al. (1995) Biol. Cell 85(1):1-7) which can be prepared by methods well known to those skilled in the art.

The polynucleotide vector constructs containing the gene encoding the angiogenic polypeptide used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA. Unlike other gene therapies techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

The polynucleotide construct containing the gene encoding the angiogenic polypeptide can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis,

ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue  
5    ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are  
10   differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. In vivo muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the injection of naked polynucleotide encoding the angiogenic polypeptide, an  
15   effective dosage amount of DNA or RNA will be in the range of from about 0.05 g/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic  
20   acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or  
25   mucous membranes of the nose. In addition, naked polynucleotide constructs containing the gene encoding the angiogenic polypeptide can be delivered to arteries during angioplasty by the catheter used in the procedure.

The dose response effects of injected polynucleotide encoding the angiogenic polypeptide in muscle in vivo is determined as follows. Suitable template DNA encoding  
30   the angiogenic polypeptide for production of mRNA coding for angiogenic polypeptide is prepared in accordance with a standard recombinant DNA methodology. The template DNA, which may be either circular or linear, is either used as naked DNA or complexed with liposomes. The quadriceps muscles of mice are then injected with various amounts of the template DNA.

35   Five to six week old female and male Balb/C mice are anesthetized by intraperitoneal injection with 0.3 ml of 2.5% Avertin. A 1.5 cm incision is made on the anterior thigh, and the quadriceps muscle is directly visualized. The template DNA

encoding the angiogenic polypeptide is injected in 0.1 ml of carrier in a 1 cc syringe through a 27 gauge needle over one minute, approximately 0.5 cm from the distal insertion site of the muscle into the knee and about 0.2 cm deep. A suture is placed over the injection site for future localization, and the skin is closed with stainless steel clips.

5 After an appropriate incubation time (e.g., 7 days) muscle extracts are prepared by excising the entire quadriceps. Every fifth 15 um cross-section of the individual quadriceps muscles is histochemically stained for angiogenic protein expression. A time course for angiogenic protein expression may be done in a similar fashion except that quadriceps from different mice are harvested at different times. Persistence of DNA  
10 encoding angiogenic polypeptide in muscle following injection may be determined by Southern blot analysis after preparing total cellular DNA and HIRT supernatants from injected and control mice. The results of the above experimentation in mice can be use to extrapolate proper dosages and other treatment parameters in humans and other animals using naked DNA encoding for angiogenic polypeptide.

#### 15 **Example 21: Angiogenic Protein Transgenic Animals.**

The angiogenic polypeptides can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, hamsters, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, e.g.,  
20 baboons, monkeys, and chimpanzees may be used to generate transgenic animals. In a specific embodiment, techniques described herein or otherwise known in the art, are used to express polypeptides of the invention in humans, as part of a gene therapy protocol.

Any technique known in the art may be used to introduce the transgene (i.e., polynucleotides of the invention) into animals to produce the founder lines of transgenic  
25 animals. Such techniques include, but are not limited to, pronuclear microinjection (Paterson et al., Appl. Microbiol. Biotechnol. 40:691-698 (1994); Carver et al., Biotechnology (NY) 11:1263-1270 (1993); Wright et al., Biotechnology (NY) 9:830-834 (1991); and Hoppe et al., U.S. Pat. No. 4,873,191 (1989)); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci., USA 82:6148-  
30 6152 (1985)), blastocysts or embryos; gene targeting in embryonic stem cells (Thompson et al., Cell 56:313-321 (1989)); electroporation of cells or embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814 (1983)); introduction of the polynucleotides of the invention using a gene gun (see, e.g., Ulmer et al., Science 259:1745 (1993); introducing nucleic acid constructs into embryonic pluripotent stem cells and transferring the stem cells back into  
35 the blastocyst; and sperm-mediated gene transfer (Lavitrano et al., Cell 57:717-723 (1989); etc. For a review of such techniques, see Gordon, "Transgenic Animals," Intl.

Rev. Cytol. 115:171-229 (1989), which is incorporated by reference herein in its entirety.

Any technique known in the art may be used to produce transgenic clones containing polynucleotides of the invention, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal, or adult cells induced to quiescence (Campbell et al., Nature 380:64-66 (1996); Wilmut et al., Nature 385:810-813 (1997)).

The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals or chimeric. The transgene may be integrated as a single transgene or as multiple copies such as in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko et al., Proc. Natl. Acad. Sci. USA 89:6232-6236 (1992)). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the polynucleotide transgene be integrated into the chromosomal site of the endogenous gene, gene targeting is preferred.

Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene in only that cell type, by following, for example, the teaching of Gu et al. (Gu et al., Science 265:103-106 (1994)). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. The contents of each of the documents recited in this paragraph is herein incorporated by reference in its entirety.

Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and reverse transcriptase-PCR (rt-PCR). Samples of transgenic gene-expressing tissue may also be evaluated immunocytochemically or immunohistochemically using antibodies specific for the transgene product.



Once the founder animals are produced, they may be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to: outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound transgenics that express the transgene at higher levels because of the effects of additive expression of each transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; and breeding to place the transgene on a distinct background that is appropriate for an experimental model of interest.

Transgenic animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of angiogenic polypeptides, studying conditions and/or disorders associated with aberrant angiogenic polypeptide expression, and in screening for compounds effective in ameliorating such conditions and/or disorders.

**Example 22: Angiogenic Protein Knock-Out Animals.**

Endogenous angiogenic protein gene expression can also be reduced by inactivating or "knocking out" the gene encoding the angiogenic protein and/or its promoter using targeted homologous recombination. (E.g., see Smithies et al., Nature 317:230-234 (1985); Thomas & Capecchi, Cell 51:503-512 (1987); Thompson et al., Cell 5:313-321 (1989); each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional polynucleotide of the invention (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous polynucleotide sequence (either the coding regions or regulatory regions of the gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express polypeptides of the invention in vivo. In another embodiment, techniques known in the art are used to generate knockouts in cells that contain, but do not express the gene of interest. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the targeted gene. Such approaches are particularly suited in research and agricultural fields where modifications to embryonic stem cells can be used to generate animal offspring with an inactive targeted gene (e.g., see Thomas & Capecchi 1987 and Thompson 1989, *supra*). However this approach can be routinely adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site in vivo using appropriate viral vectors that will be apparent to those of skill in the art.

In further embodiments of the invention, cells that are genetically engineered to express the polypeptides of the invention, or alternatively, that are genetically engineered not to express the polypeptides of the invention (e.g., knockouts) are administered to a patient in vivo. Such cells may be obtained from the patient (i.e., animal, including human) or an MHC compatible donor and can include, but are not limited to fibroblasts, bone marrow cells, blood cells (e.g., lymphocytes), adipocytes, muscle cells, endothelial cells etc. The cells are genetically engineered in vitro using recombinant DNA techniques to introduce the coding sequence of polypeptides of the invention into the cells, or alternatively, to disrupt the coding sequence and/or endogenous regulatory sequence associated with the polypeptides of the invention, e.g., by transduction (using viral vectors, and preferably vectors that integrate the transgene into the cell genome) or transfection procedures, including, but not limited to, the use of plasmids, cosmids, YACs, naked DNA, electroporation, liposomes, etc. The coding sequence of the polypeptides of the invention can be placed under the control of a strong constitutive or inducible promoter or promoter/enhancer to achieve expression, and preferably secretion, of the angiogenic polypeptides. The engineered cells which express and preferably secrete the polypeptides of the invention can be introduced into the patient systemically, e.g., in the circulation, or intraperitoneally.

Alternatively, the cells can be incorporated into a matrix and implanted in the body, e.g., genetically engineered fibroblasts can be implanted as part of a skin graft; genetically engineered endothelial cells can be implanted as part of a lymphatic or vascular graft. (See, for example, Anderson et al. U.S. Patent No. 5,399,349; and Mulligan & Wilson, U.S. Patent No. 5,460,959 each of which is incorporated by reference herein in its entirety).

When the cells to be administered are non-autologous or non-MHC compatible cells, they can be administered using well known techniques which prevent the development of a host immune response against the introduced cells. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

Knock-out animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of angiogenic polypeptides, studying conditions and/or disorders associated with aberrant angiogenic protein expression, and in screening for compounds effective in ameliorating such conditions and/or disorders.

**Example 23: Angiogenic Polypeptide Biological Effects**

**Fibroblast and endothelial cell assays.** Human lung fibroblasts are obtained from Clonetics (San Diego, CA) and maintained in growth media from Clonetics. Dermal microvascular endothelial cells are obtained from Cell Applications (San Diego, CA). For proliferation assays, the human lung fibroblasts and dermal microvascular endothelial cells can be cultured at 5,000 cells/well in a 96-well plate for one day in growth medium. The cells are then incubated for one day in 0.1% BSA basal medium. After replacing the medium with fresh 0.1% BSA medium, the cells are incubated with the test proteins for 3 days. Alamar Blue (Alamar Biosciences, Sacramento, CA) is added to each well to a final concentration of 10%. The cells are incubated for 4 hr. Cell viability is measured by reading in a CytoFluor fluorescence reader. For the PGE<sub>2</sub> assays, the human lung fibroblasts are cultured at 5,000 cells/well in a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or angiogenic polypeptide with or without IL-1 $\alpha$  for 24 hours. The supernatants are collected and assayed for PGE<sub>2</sub> by EIA kit (Cayman, Ann Arbor, MI). For the IL-6 assays, the human lung fibroblasts are cultured at 5,000 cells/well in a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or the angiogenic polypeptide with or without IL-1 $\alpha$  for 24 hours. The supernatants are collected and assayed for IL-6 by ELISA kit (Endogen, Cambridge, MA).

Human lung fibroblasts are cultured with FGF-2 or the angiogenic polypeptide for 3 days in basal medium before the addition of Alamar Blue to assess effects on growth of the fibroblasts. FGF-2 should show a stimulation at 10 - 2500 ng/ml which can be used to compare stimulation with the angiogenic polypeptide.

**Example 24: The Effect of the Angiogenic Polypeptide on the Growth of Vascular Endothelial Cells**

On day 1, human umbilical vein endothelial cells (HUVEC) are seeded at  $2.5 \times 10^4$  cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, and 50 units/ml endothelial cell growth supplements (ECGS, Biotechnology, Inc.). On day 2, the medium is replaced with M199 containing 10% FBS, 8 units/ml heparin, angiogenic protein of, and positive controls, such as basic FGF (bFGF) are added, at varying concentrations. On days 4 and 6, the medium is replaced. On day 8, cell number is determined with a Coulter Counter.

An increase in the number of HUVEC cells indicates that the angiogenic polypeptide may proliferate vascular endothelial cells.

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

**Example 25: Stimulatory Effect of the Angiogenic Polypeptide on the Proliferation of Vascular Endothelial Cells**

For evaluation of mitogenic activity of growth factors, the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium) assay with the electron coupling reagent PMS (phenazine methosulfate) was performed (CellTiter 96 AQ, Promega). Cells are seeded in a 96-well plate (5,000 cells/well) in 0.1 mL serum-supplemented medium and are allowed to attach overnight. After serum-starvation for 12 hours in 0.5% FBS, conditions (bFGF, VEGF<sub>165</sub> or the angiogenic polypeptide in 0.5% FBS) with or without Heparin (8 U/ml) are added to wells for 48 hours. 20 mg of MTS/PMS mixture (1:0.05) are added per well and allowed to incubate for 1 hour at 37°C before measuring the absorbance at 490 nm in an ELISA plate reader. Background absorbance from control wells (some media, no cells) is subtracted, and seven wells are performed in parallel for each condition. See, Leak *et al. In Vitro Cell. Dev. Biol.* 30A:512-518 (1994).

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

**Example 26: Inhibition of PDGF-induced Vascular Smooth Muscle Cell Proliferation Stimulatory Effect**

HAoSMC proliferation can be measured, for example, by BrdUrd incorporation. Briefly, subconfluent, quiescent cells grown on the 4-chamber slides are transfected with CRP or FITC-labeled AT2-3LP. Then, the cells are pulsed with 10% calf serum and 6 mg/ml BrdUrd. After 24 h, immunocytochemistry is performed by using BrdUrd Staining Kit (Zymed Laboratories). In brief, the cells are incubated with the biotinylated mouse anti-BrdUrd antibody at 4 °C for 2 h after being exposed to denaturing solution and then incubated with the streptavidin-peroxidase and diaminobenzidine.

After counterstaining with hematoxylin, the cells are mounted for microscopic examination, and the BrdUrd-positive cells are counted. The BrdUrd index is calculated

as a percent of the BrdUrd-positive cells to the total cell number. In addition, the simultaneous detection of the BrdUrd staining (nucleus) and the FITC uptake (cytoplasm) is performed for individual cells by the concomitant use of bright field illumination and dark field-UV fluorescent illumination. See, Hayashida et al., J. Biol. Chem.

5 6:271(36):21985-21992 (1996).

The studies described in this example tested activity in angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

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#### **Example 27: Stimulation of Endothelial Migration**

This example will be used to explore the possibility that the angiogenic polypeptide may stimulate lymphatic endothelial cell migration.

15 Endothelial cell migration assays are performed using a 48 well microchemotaxis chamber (Neuroprobe Inc., Cabin John, MD; Falk, W., et al., J. Immunological Methods 1980;33:239-247). Polyvinylpyrrolidone-free polycarbonate filters with a pore size of 8  $\mu$ m (Nucleopore Corp. Cambridge, MA) are coated with 0.1% gelatin for at least 6 hours at room temperature and dried under sterile air. Test substances are diluted to appropriate concentrations in M199 supplemented with 0.25% bovine serum albumin (BSA), and 25  $\mu$ l of the final dilution is placed in the lower chamber of the modified  
20 Boyden apparatus. Subconfluent, early passage (2-6) HUVEC or BMEC cultures are washed and trypsinized for the minimum time required to achieve cell detachment. After placing the filter between lower and upper chamber,  $2.5 \times 10^5$  cells suspended in 50  $\mu$ l M199 containing 1% FBS are seeded in the upper compartment. The apparatus is then  
25 incubated for 5 hours at 37°C in a humidified chamber with 5% CO<sub>2</sub> to allow cell migration. After the incubation period, the filter is removed and the upper side of the filter with the non-migrated cells is scraped with a rubber policeman. The filters are fixed with methanol and stained with a Giemsa solution (Diff-Quick, Baxter, McGraw Park, IL). Migration is quantified by counting cells of three random high-power fields (40x)  
30 in each well, and all groups are performed in quadruplicate.

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

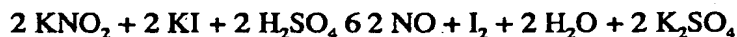
35

**Example 28: Stimulation of Nitric Oxide Production by Endothelial Cells**

Nitric oxide released by the vascular endothelium is believed to be a mediator of vascular endothelium relaxation. Thus, angiogenic polypeptide activity can be assayed by determining nitric oxide production by endothelial cells in response to angiogenic polypeptide.

Nitric oxide is measured in 96-well plates of confluent microvascular endothelial cells after 24 hours starvation and a subsequent 4 hr exposure to various levels of a positive control and angiogenic polypeptide. Nitric oxide in the medium is determined by use of the Griess reagent to measure total nitrite after reduction of nitric oxide-derived nitrate by nitrate reductase. The effect of angiogenic polypeptide on nitric oxide release is examined on HUVEC.

Briefly, NO release from cultured HUVEC monolayer is measured with a NO-specific polarographic electrode connected to a NO meter (Iso-NO, World Precision Instruments Inc.) (1049). Calibration of the NO elements is performed according to the following equation:



The standard calibration curve is obtained by adding graded concentrations of  $\text{KNO}_2$  (0, 5, 10, 25, 50, 100, 250, and 500 nmol/L) into the calibration solution containing KI and  $\text{H}_2\text{SO}_4$ . The specificity of the Iso-NO electrode to NO is previously determined by measurement of NO from authentic NO gas (1050). The culture medium is removed and HUVECs are washed twice with Dulbecco's phosphate buffered saline. The cells are then bathed in 5 ml of filtered Krebs-Henseleit solution in 6-well plates, and the cell plates are kept on a slide warmer (Lab Line Instruments Inc.) To maintain the temperature at 37°C. The NO sensor probe is inserted vertically into the wells, keeping the tip of the electrode 2 mm under the surface of the solution, before addition of the different conditions. S-nitroso acetyl penicillamin (SNAP) is used as a positive control. The amount of released NO is expressed as picomoles per  $1 \times 10^6$  endothelial cells. All values reported are means of four to six measurements in each group (number of cell culture wells). See, Leak *et al. Biochem. and Biophys. Res. Comm.* 217:96-105 (1995).

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

**Example 29: Effect of the Angiogenic Polypeptide on Cord Formation in Angiogenesis**

Another step in angiogenesis is cord formation, marked by differentiation of endothelial cells. This bioassay measures the ability of microvascular endothelial cells to form capillary-like structures (hollow structures) when cultured *in vitro*.

CADMEC (microvascular endothelial cells) are purchased from Cell Applications, Inc. as proliferating (passage 2) cells and are cultured in Cell Applications' CADMEC Growth Medium and used at passage 5. For the *in vitro* angiogenesis assay, the wells of a 48-well cell culture plate are coated with Cell Applications' Attachment Factor Medium (200 µl/well) for 30 min. at 37°C. CADMEC are seeded onto the coated wells at 7,500 cells/well and cultured overnight in Growth Medium. The Growth Medium is then replaced with 300 µg Cell Applications' Chord Formation Medium containing control buffer or the angiogenic polypeptide (0.1 to 100 ng/ml) and the cells are cultured for an additional 48 hr. The numbers and lengths of the capillary-like chords are quantitated through use of the Boeckeler VIA-170 video image analyzer. All assays are done in triplicate.

Commercial (R&D) VEGF (50 ng/ml) is used as a positive control.  $\beta$ -estradiol (1 ng/ml) is used as a negative control. The appropriate buffer (without protein) is also utilized as a control. The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

**Example 30: Angiogenic Effect on Chick Chorioallantoic Membrane**

Chick chorioallantoic membrane (CAM) is a well-established system to examine angiogenesis. Blood vessel formation on CAM is easily visible and quantifiable. The ability of the angiogenic polypeptide to stimulate angiogenesis in CAM can be examined. Fertilized eggs of the White Leghorn chick (*Gallus gallus*) and the Japanese quail (*Coturnix coturnix*) are incubated at 37.8°C and 80% humidity. Differentiated CAM of 16-day-old chick and 13-day-old quail embryos is studied with the following methods. On Day 4 of development, a window is made into the egg shell of chick eggs. The embryos are checked for normal development and the eggs sealed with cellotape. They are further incubated until Day 13. Thermanox coverslips (Nunc, Naperville, IL) are cut into disks of about 5 mm in diameter. Sterile and salt-free growth factors are dissolved in distilled water and about 3.3 µg/5 ml are pipetted on the disks. After air-drying, the inverted disks are applied on CAM. After 3 days, the specimens are fixed in 3% glutaraldehyde and 2% formaldehyde and rinsed in 0.12 M sodium cacodylate buffer. They are

photographed with a stereo microscope [Wild M8] and embedded for semi- and ultrathin sectioning as described above. Controls are performed with carrier disks alone.

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

### **Example 31: Angiogenesis Assay Using a Matrigel Implant in Mouse**

*In vivo* angiogenesis assay of the angiogenic polypeptide measures the ability of an existing capillary network to form new vessels in an implanted capsule of murine extracellular matrix material (Matrigel). The protein is mixed with the liquid Matrigel at 4 degree C and the mixture is then injected subcutaneously in mice where it solidifies. After 7 days, the solid "plug" of Matrigel is removed and examined for the presence of new blood vessels. Matrigel is purchased from Becton Dickinson Labware/Collaborative Biomedical Products.

When thawed at 4 degree C the Matrigel material is a liquid. The Matrigel is mixed with the angiogenic polypeptide at 150 ng/ml at 4 degree C and drawn into cold 3 ml syringes. Female C57Bl/6 mice approximately 8 weeks old are injected with the mixture of Matrigel and experimental protein at 2 sites at the midventral aspect of the abdomen (0.5 ml/site). After 7 days, the mice are sacrificed by cervical dislocation, the Matrigel plugs are removed and cleaned (i.e., all clinging membranes and fibrous tissue is removed). Replicate whole plugs are fixed in neutral buffered 10% formaldehyde, embedded in paraffin and used to produce sections for histological examination after staining with Masson's Trichrome. Cross sections from 3 different regions of each plug are processed. Selected sections are stained for the presence of vWF. The positive control for this assay is bovine basic FGF (150 ng/ml). Matrigel alone is used to determine basal levels of angiogenesis.

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

### **Example 32: Rescue of Ischemia in Rabbit Lower Limb Model**

To study the *in vivo* effects of the angiogenic polypeptide on ischemia, a rabbit hindlimb ischemia model is created by surgical removal of one femoral arteries as described previously (Takeshita, S. *et al.*, *Am J. Pathol* 147:1649-1660 (1995)). The



excision of the femoral artery results in retrograde propagation of thrombus and occlusion of the external iliac artery. Consequently, blood flow to the ischemic limb is dependent upon collateral vessels originating from the internal iliac artery (Takeshita, S. *et al. Am J. Pathol* 147:1649-1660 (1995)). An interval of 10 days is allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. At 10 day post-operatively (day 0), after performing a baseline angiogram, the internal iliac artery of the ischemic limb is transfected with 500 mg naked expression plasmid encoding the angiogenic polypeptide by arterial gene transfer technology using a hydrogel-coated balloon catheter as described (Riessen, R. *et al. Hum Gene Ther.* 4:749-758 (1993); Leclerc, G. *et al. J. Clin. Invest.* 90: 936-944 (1992)). When DNA encoding the angiogenic polypeptide is used in the treatment, a single bolus of 500 mg of the angiogenic protein or control is delivered into the internal iliac artery of the ischemic limb over a period of 1 min. through an infusion catheter. On day 30, various parameters are measured in these rabbits: (a) BP ratio - The blood pressure ratio of systolic pressure of the ischemic limb to that of normal limb; (b) Blood Flow and Flow Reserve - Resting FL: the blood flow during undilated condition and Max FL: the blood flow during fully dilated condition (also an indirect measure of the blood vessel amount) and Flow Reserve is reflected by the ratio of max FL: resting FL; (c) Angiographic Score - This is measured by the angiogram of collateral vessels. A score is determined by the percentage of circles in an overlaying grid that with crossing opacified arteries divided by the total number in the rabbit thigh; (d) Capillary density - The number of collateral capillaries determined in light microscopic sections taken from hindlimbs.

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

### **Example 33: Effect of the Angiogenic Protein on Vasodilation**

Since dilation of vascular endothelium is important in reducing blood pressure, the ability of the angiogenic polypeptide to affect the blood pressure in spontaneously hypertensive rats (SHR) is examined. Increasing doses (0, 10, 30, 100, 300, and 900 mg/kg) of the angiogenic polypeptide is administered to 13-14 week old spontaneously hypertensive rats (SHR). Data are expressed as the mean  $\pm$  SEM. Statistical analysis are performed with a paired t-test and statistical significance is defined as  $p < 0.05$  vs. the response to buffer alone.

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the

activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

**Example 34: Rat Ischemic Skin Flap Model**

5 The evaluation parameters include skin blood flow, skin temperature, and factor VIII immunohistochemistry or endothelial alkaline phosphatase reaction. Angiogenic protein expression, during the skin ischemia, is studied using in situ hybridization.

The study in this model is divided into three parts as follows:

- 10 a) Ischemic skin
- b) Ischemic skin wounds
- c) Normal wounds

The experimental protocol includes:

- 15 a) Raising a 3x4 cm, single pedicle full-thickness random skin flap (myocutaneous flap over the lower back of the animal).
- b) An excisional wounding (4-6 mm in diameter) in the ischemic skin (skin-flap).
- c) Topical treatment with the angiogenic polypeptide of the excisional wounds (day 0, 1, 2, 3, 4 post-wounding) at the following various dosage ranges: 1mg to 100 mg.
- d) Harvesting the wound tissues at day 3, 5, 7, 10, 14 and 21 post-wounding for histological, immunohistochemical, and in situ studies.

20 The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

25 **Example 35: Peripheral Arterial Disease Model**

Angiogenic therapy using the angiogenic polypeptide is a novel therapeutic strategy to obtain restoration of blood flow around the ischemia in case of peripheral arterial diseases. The experimental protocol includes:

- 30 a) One side of the femoral artery is ligated to create ischemic muscle of the hindlimb, the other side of hindlimb serves as a control.
- b) Angiogenic protein, in a dosage range of 20 mg - 500 mg, is delivered intravenously and/or intramuscularly 3 times (perhaps more) per week for 2-3 weeks.
- c) The ischemic muscle tissue is collected after ligation of the femoral artery at 1, 2, and 3 weeks for the analysis of the angiogenic protein expression and histology.
- 35 Biopsy is also performed on the other side of normal muscle of the contralateral hindlimb.

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

**Example 36: Ischemic Myocardial Disease Model**

The angiogenic polypeptide is evaluated as a potent mitogen capable of stimulating the development of collateral vessels, and restructuring new vessels after coronary artery occlusion. Alteration of the angiogenic protein expression is investigated in situ. The experimental protocol includes:

- a) The heart is exposed through a left-side thoracotomy in the rat. Immediately, the left coronary artery is occluded with a thin suture (6-0) and the thorax is closed.
- b) Angiogenic protein, in a dosage range of 20 mg - 500 mg, is delivered intravenously and/or intramuscularly 3 times (perhaps more) per week for 2-4 weeks.
- c) Thirty days after the surgery, the heart is removed and cross-sectioned for morphometric and in situ analyzes.

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

**Example 37: Rat Corneal Wound Healing Model**

This animal model shows the effect of the angiogenic polypeptide on neovascularization. The experimental protocol includes:

- a) Making a 1-1.5 mm long incision from the center of cornea into the stromal layer.
- b) Inserting a spatula below the lip of the incision facing the outer corner of the eye.
- c) Making a pocket (its base is 1-1.5 mm from the edge of the eye).
- d) Positioning a pellet, containing 50ng- 5ug of the angiogenic polypeptide, within the pocket.
- e) Angiogenic polypeptide treatment can also be applied topically to the corneal wounds in a dosage range of 20mg - 500mg (daily treatment for five days).

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

### **Example 38: Diabetic Mouse and Glucocorticoid-Impaired Wound Healing Models**

#### **A. Diabetic db+/db+ Mouse Model.**

5 To demonstrate that the angiogenic polypeptide can accelerate the healing process, the genetically diabetic mouse model of wound healing is used. The full thickness wound healing model in the db+/db+ mouse is a well characterized, clinically relevant and reproducible model of impaired wound healing. Healing of the diabetic wound is dependent on formation of granulation tissue and re-epithelialization rather than  
10 contraction (Gartner, M.H. *et al.*, *J. Surg. Res.* 52:389 (1992); Greenhalgh, D.G. *et al.*, *Am. J. Pathol.* 136:1235 (1990)).

The diabetic animals have many of the characteristic features observed in Type II diabetes mellitus. Homozygous (db+/db+) mice are obese in comparison to their normal heterozygous (db+/-) littermates. Mutant diabetic (db+/db+) mice have a single  
15 autosomal recessive mutation on chromosome 4 (db+) (Coleman *et al.* *Proc. Natl. Acad. Sci. USA* 77:283-293 (1982)). Animals show polyphagia, polydipsia and polyuria. Mutant diabetic mice (db+/db+) have elevated blood glucose, increased or normal insulin levels, and suppressed cell-mediated immunity (Mandel *et al.*, *J. Immunol.* 120:1375 (1978); Debray-Sachs, M. *et al.*, *Clin. Exp. Immunol.* 51(1):1-7 (1983); Leiter *et al.*,  
20 *Am. J. of Pathol.* 114:46-55 (1985)). Peripheral neuropathy, myocardial complications, and microvascular lesions, basement membrane thickening and glomerular filtration abnormalities have been described in these animals (Norido, F. *et al.*, *Exp. Neurol.* 83(2):221-232 (1984); Robertson *et al.*, *Diabetes* 29(1):60-67 (1980); Giacomelli *et al.*, *Lab Invest.* 40(4):460-473 (1979); Coleman, D.L., *Diabetes* 31 (Suppl):1-6 (1982)).  
25 These homozygous diabetic mice develop hyperglycemia that is resistant to insulin analogous to human type II diabetes (Mandel *et al.*, *J. Immunol.* 120:1375-1377 (1978)).

The characteristics observed in these animals suggests that healing in this model may be similar to the healing observed in human diabetes (Greenhalgh, *et al.*, *Am. J. of Pathol.* 136:1235-1246 (1990)).

30 Genetically diabetic female C57BL/KsJ (db+/db+) mice and their non-diabetic (db+/-) heterozygous littermates are used in this study (Jackson Laboratories). The animals are purchased at 6 weeks of age and are 8 weeks old at the beginning of the study. Animals are individually housed and received food and water ad libitum. All manipulations are performed using aseptic techniques. The experiments are conducted  
35 according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

Wounding protocol is performed according to previously reported methods (Tsuboi, R. and Rifkin, D.B., *J. Exp. Med.* 172:245-251 (1990)). Briefly, on the day of wounding, animals are anesthetized with an intraperitoneal injection of Avertin (0.01 mg/mL), 2,2,2-tribromoethanol and 2-methyl-2-butanol dissolved in deionized water.

5 The dorsal region of the animal is shaved and the skin washed with 70% ethanol solution and iodine. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is then created using a Keyes tissue punch. Immediately following wounding, the surrounding skin is gently stretched to eliminate wound expansion. The wounds are left open for the duration of the experiment. Application of the treatment is  
10 given topically for 5 consecutive days commencing on the day of wounding. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of surgery and at two day intervals thereafter. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically  
15 using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

The angiogenic polypeptide is administered using a range different doses of the angiogenic polypeptide, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

20 Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology and immunohistochemistry. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing. Three groups of 10 animals each (5 diabetic and 5 non-diabetic controls) are  
25 evaluated:

- 1) Vehicle placebo control
- 2) The angiogenic polypeptide

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total square area of the wound. Contraction is then estimated by  
30 establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is 64mm<sup>2</sup>, the corresponding size of the dermal punch. Calculations are made using the following formula: [Open area on day 8] - [Open area on day 1] / [Open area on day 1] Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface  
35 (5mm) and cut using a Reichert-Jung microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds are used to assess whether the healing process and the morphologic

appearance of the repaired skin is altered by treatment with angiogenic polypeptide. This assessment included verification of the presence of cell accumulation, inflammatory cells, capillaries, fibroblasts, re-epithelialization and epidermal maturity (Greenhalgh, D.G. *et al.*, *Am. J. Pathol.* 136:1235 (1990)). A calibrated lens micrometer is used by a blinded observer.

Tissue sections are also stained immunohistochemically with a polyclonal rabbit anti-human keratin antibody using ABC Elite detection system. Human skin is used as a positive tissue control while non-immune IgG is used as a negative control. Keratinocyte growth is determined by evaluating the extent of reepithelialization of the wound using a calibrated lens micrometer.

Proliferating cell nuclear antigen/cyclin (PCNA) in skin specimens is demonstrated by using anti-PCNA antibody (1:50) with an ABC Elite detection system. Human colon cancer served as a positive tissue control and human brain tissue is used as a negative tissue control. Each specimen included a section with omission of the primary antibody and substitution with non-immune mouse IgG. Ranking of these sections is based on the extent of proliferation on a scale of 0-8, the lower side of the scale reflecting slight proliferation to the higher side reflecting intense proliferation. Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

#### **B. Steroid Impaired Rat Model**

The inhibition of wound healing by steroids has been well documented in various *in vitro* and *in vivo* systems (Wahl, S.M. Glucocorticoids and Wound healing. In: Anti-Inflammatory Steroid Action: Basic and Clinical Aspects. 280-302 (1989); Wahl, S.M.*et al.*, *J. Immunol.* 115: 476-481 (1975); Werb, Z. *et al.*, *J. Exp. Med.* 147:1684-1694 (1978)). Glucocorticoids retard wound healing by inhibiting angiogenesis, decreasing vascular permeability ( Ebert, R.H., *et al.*, *Am. Intern. Med.* 37:701-705 (1952)), fibroblast proliferation, and collagen synthesis (Beck, L.S. *et al.*, *Growth Factors.* 5: 295-304 (1991); Haynes, B.F. *et al.*, *J. Clin. Invest.* 61: 703-797 (1978)) and producing a transient reduction of circulating monocytes (Haynes, B.F., *et al.*, *J. Clin. Invest.* 61: 703-797 (1978); Wahl, S. M., "Glucocorticoids and wound healing", In: Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989)). The systemic administration of steroids to impaired wound healing is a well establish phenomenon in rats (Beck, L.S. *et al.*, *Growth Factors.* 5: 295-304 (1991); Haynes, B.F., *et al.*, *J. Clin. Invest.* 61: 703-797 (1978); Wahl, S. M., "Glucocorticoids and wound healing", In: Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989); Pierce, G.F. *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 2229-2233 (1989)).

To demonstrate that the angiogenic polypeptide can accelerate the healing process, the effects of multiple topical applications of the angiogenic polypeptide on full thickness excisional skin wounds in rats in which healing has been impaired by the systemic administration of methylprednisolone is assessed.

5        Young adult male Sprague Dawley rats weighing 250-300 g (Charles River Laboratories) are used in this example. The animals are purchased at 8 weeks of age and are 9 weeks old at the beginning of the study. The healing response of rats is impaired by the systemic administration of methylprednisolone (17mg/kg/rat intramuscularly) at the time of wounding. Animals are individually housed and received food and water *ad*  
10 *libitum*. All manipulations are performed using aseptic techniques. This study is conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

The wounding protocol is followed according to section A, above. On the day of  
15 wounding, animals are anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The dorsal region of the animal is shaved and the skin washed with 70% ethanol and iodine solutions. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is created using a Keyes tissue punch. The wounds are left open for the duration of the experiment. Applications of the  
20 testing materials are given topically once a day for 7 consecutive days commencing on the day of wounding and subsequent to methylprednisolone administration. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of wounding and at the end of treatment. Wound closure is determined by daily  
25 measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

The angiogenic polypeptide is administered using at a range different doses of the angiogenic polypeptide, from 4mg to 500mg per wound per day for 8 days in vehicle.  
30 Vehicle control groups received 50mL of vehicle solution. Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

35        Four groups of 10 animals each (5 with methylprednisolone and 5 without glucocorticoid) are evaluated:

1) Untreated group

2) Vehicle placebo control

3) The angiogenic polypeptide treated groups.

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total area of the wound. Closure is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is 64mm<sup>2</sup>, the corresponding size of the dermal punch. Calculations are made using the following formula:

$$[\text{Open area on day 8}] - [\text{Open area on day 1}] / [\text{Open area on day 1}]$$

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using an Olympus microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds allows assessment of whether the healing process and the morphologic appearance of the repaired skin is improved by treatment with the angiogenic polypeptide. A calibrated lens micrometer is used by a blinded observer to determine the distance of the wound gap.

Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

#### **Example 39: Lymphedema Animal Model**

The purpose of this experimental approach is to create an appropriate and consistent lymphedema model for testing the therapeutic effects of the angiogenic polypeptide in lymphangiogenesis and re-establishment of the lymphatic circulatory system in the rat hind limb. Effectiveness is measured by swelling volume of the affected limb, quantification of the amount of lymphatic vasculature, total blood plasma protein, and histopathology. Acute lymphedema is observed for 7-10 days. Perhaps more importantly, the chronic progress of the edema is followed for up to 3-4 weeks. Prior to beginning surgery, blood sample is drawn for protein concentration analysis. Male rats weighing approximately ~350g are dosed with Pentobarbital. Subsequently, the right legs are shaved from knee to hip. The shaved area is swabbed with gauze soaked in 70% EtOH. Blood is drawn for serum total protein testing. Circumference and volumetric measurements are made prior to injecting dye into paws after marking 2 measurement levels (0.5 cm above heel, at mid-pt of dorsal paw). The intradermal dorsum of both right and left paws are injected with 0.05 ml of 1% Evan's Blue.



Circumference and volumetric measurements are then made following injection of dye into paws.

Using the knee joint as a landmark, a mid-leg inguinal incision is made circumferentially allowing the femoral vessels to be located. Forceps and hemostats are used to dissect and separate the skin flaps. After locating the femoral vessels, the lymphatic vessel that runs along side and underneath the vessel(s) is located. The main lymphatic vessels in this area are then electrically coagulated or suture ligated.

Using a microscope, muscles in back of the leg (near the semitendinosus and adductors) are bluntly dissected. The popliteal lymph node is then located. The 2 proximal and 2 distal lymphatic vessels and distal blood supply of the popliteal node are then and ligated by suturing. The popliteal lymph node, and any accompanying adipose tissue, is then removed by cutting connective tissues.

Care is taken to control any mild bleeding resulting from this procedure. After lymphatics are occluded, the skin flaps are sealed by using liquid skin (Vetbond) (AJ Buck). The separated skin edges are sealed to the underlying muscle tissue while leaving a gap of ~0.5 cm around the leg. Skin also may be anchored by suturing to underlying muscle when necessary.

To avoid infection, animals are housed individually with mesh (no bedding). Recovering animals are checked daily through the optimal edematous peak, which typically occurred by day 5-7. The plateau edematous peak are then observed. To evaluate the intensity of the lymphedema, the circumference and volumes of 2 designated places on each paw before operation and daily for 7 days are measured. The effect plasma proteins on lymphedema is determined and whether protein analysis is a useful testing perimeter is also investigated. The weights of both control and edematous limbs are evaluated at 2 places. Analysis is performed in a blind manner.

**Circumference Measurements:** Under brief gas anesthetic to prevent limb movement, a cloth tape is used to measure limb circumference. Measurements are done at the ankle bone and dorsal paw by 2 different people then those 2 readings are averaged. Readings are taken from both control and edematous limbs.

**Volumetric Measurements:** On the day of surgery, animals are anesthetized with Pentobarbital and are tested prior to surgery. For daily volumetrics animals are under brief halothane anesthetic (rapid immobilization and quick recovery), both legs are shaved and equally marked using waterproof marker on legs. Legs are first dipped in water, then dipped into instrument to each marked level then measured by Buxco edema software(Chen/Victor). Data is recorded by one person, while the other is dipping the limb to marked area.

**Blood-plasma protein measurements:** Blood is drawn, spun, and serum separated prior to surgery and then at conclusion for total protein and Ca<sup>2+</sup> comparison.

**Limb Weight Comparison:** After drawing blood, the animal is prepared for tissue collection. The limbs are amputated using a quillitine, then both experimental and control legs are cut at the ligature and weighed. A second weighing is done as the tibio-cacaneal joint is disarticulated and the foot is weighed.

**Histological Preparations:** The transverse muscle located behind the knee (popliteal) area is dissected and arranged in a metal mold, filled with freezeGel, dipped into cold methylbutane, placed into labeled sample bags at - 80EC until sectioning. Upon sectioning, the muscle is observed under fluorescent microscopy for lymphatics. The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

**Example 40: Suppression of TNF alpha-induced adhesion molecule expression by the Angiogenic Polypeptide**

The recruitment of lymphocytes to areas of inflammation and angiogenesis involves specific receptor-ligand interactions between cell surface adhesion molecules (CAMs) on lymphocytes and the vascular endothelium. The adhesion process, in both normal and pathological settings, follows a multi-step cascade that involves intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) expression on endothelial cells (EC). The expression of these molecules and others on the vascular endothelium determines the efficiency with which leukocytes may adhere to the local vasculature and extravasate into the local tissue during the development of an inflammatory response. The local concentration of cytokines and growth factor participate in the modulation of the expression of these CAMs.

Tumor necrosis factor alpha (TNF- $\alpha$ ), a potent proinflammatory cytokine, is a stimulator of all three CAMs on endothelial cells and may be involved in a wide variety of inflammatory responses, often resulting in a pathological outcome.

The potential of angiogenic polypeptide to mediate a suppression of TNF- $\alpha$  induced CAM expression can be examined. A modified ELISA assay which uses ECs as a solid phase absorbent is employed to measure the amount of CAM expression on TNF- $\alpha$  treated ECs when co-stimulated with a member of the angiogenic family of proteins. To perform the experiment, human umbilical vein endothelial cell (HUVEC) cultures are obtained from pooled cord harvests and maintained in growth medium (EGM-2;

Clonetics, San Diego, CA) supplemented with 10% FCS and 1% penicillin/streptomycin in a 37 degree C humidified incubator containing 5% CO<sub>2</sub>. HUVECs are seeded in 96-well plates at concentrations of  $1 \times 10^4$  cells/well in EGM medium at 37 degree C for 18-24 hrs or until confluent. The monolayers are subsequently washed 3 times with a serum-free solution of RPMI-1640 supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, and treated with a given cytokine and/or growth factor(s) for 24 h at 37 degree C. Following incubation, the cells are then evaluated for CAM expression.

Human Umbilical Vein Endothelial cells (HUVECs) are grown in a standard 96 well plate to confluence. Growth medium is removed from the cells and replaced with 90 ul of 199 Medium (10% FBS). Samples for testing and positive or negative controls are added to the plate in triplicate (in 10 ul volumes). Plates are incubated at 37 degree C for either 5 h (selectin and integrin expression) or 24 h (integrin expression only). Plates are aspirated to remove medium and 100  $\mu$ l of 0.1% paraformaldehyde-PBS(with Ca<sup>++</sup> and Mg<sup>++</sup>) is added to each well. Plates are held at 4°C for 30 mins. Fixative is then removed from the wells and wells are washed 1X with PBS(+Ca,Mg)+0.5% BSA and drained. Do not allow the wells to dry. Add 10  $\mu$ l of diluted primary antibody to the test and control wells. Anti-ICAM-1-Biotin, Anti-VCAM-1-Biotin and Anti-E-selectin-Biotin are used at a concentration of 10  $\mu$ g/ml (1:10 dilution of 0.1 mg/ml stock antibody). Cells are incubated at 37°C for 30 min. in a humidified environment. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA.

Then add 20  $\mu$ l of diluted ExtrAvidin-Alkaline Phosphatase (1:5,000 dilution) to each well and incubated at 37°C for 30 min. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA. 1 tablet of p-Nitrophenol Phosphate pNPP is dissolved in 5 ml of glycine buffer (pH 10.4). 100  $\mu$ l of pNPP substrate in glycine buffer is added to each test well. Standard wells in triplicate are prepared from the working dilution of the ExtrAvidin-Alkaline Phosphatase in glycine buffer: 1:5,000 ( $10^0$ ) >  $10^{-0.5}$  >  $10^{-1}$  >  $10^{-1.5}$ . 5  $\mu$ l of each dilution is added to triplicate wells and the resulting AP content in each well is 5.50 ng, 1.74 ng, 0.55 ng, 0.18 ng. 100  $\mu$ l of pNPP reagent must then be added to each of the standard wells. The plate must be incubated at 37°C for 4h. A volume of 50  $\mu$ l of 3M NaOH is added to all wells. The results are quantified on a plate reader at 405 nm. The background subtraction option is used on blank wells filled with glycine buffer only. The template is set up to indicate the concentration of AP-conjugate in each standard well [ 5.50 ng; 1.74 ng; 0.55 ng; 0.18 ng]. Results are indicated as amount of bound AP-conjugate in each sample.

The studies described in this example tested activity in the angiogenic protein. However, one skilled in the art could easily modify the exemplified studies to test the

activity of polynucleotides encoding the angiogenic polypeptide (e.g., gene therapy), agonists, and/or antagonists of the angiogenic polypeptide.

5 **Example 41: Mouse Immunization for Monoclonal Antibody Production**

Animals are individually housed and received food and water ad libitum. All manipulations are performed using aseptic techniques. The experiments are conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory  
10 Animals.

Dilute concentration of protein in 350  $\mu$ ls of phosphate buffered solution (PBS), or other neutral buffer, to a final protein concentration of 0.43 mg/ml. With 0.35 mls. Freund's Complete Adjuvant, emulsify the adjuvant and protein solution for a period of ten minutes using two glass 3 cc syringes and a three way disposable stopcock (Baxter  
15 Cat. No. 2C6240). To test emulsion for quality, place 50  $\mu$ ls of the emulsion onto the surface of cold water in a beaker. If the emulsion does not remain as an intact white droplet, then further mixing is required.

Draw all of the emulsion into one syringe, and using a 27 gauge needle, inject mouse subcutaneously with a total of 200  $\mu$ ls of emulsion distributed among 4-8 sites  
20 including axillary and inguinal areas, the back of the neck, and along the back.

Following two to three weeks, repeat the above injection substituting Freund's Incomplete Adjuvant (as opposed to Freund's Complete Adjuvant).

Following an additional two to three weeks, a third injection is given as outlined above, making sure to use Freund's Incomplete Adjuvant.

25 Ten to Fourteen days following the third injection, obtain 100-200  $\mu$ ls of blood from the mouse by tail vein bleed. Incubate the blood at 37°C for 60 minutes, and then allow to cool overnight at 4°C. Following incubation at 4°C, centrifuge the blood for ten minutes. Transfer the serum to a new tube, and test for mouse serum titer. If titer is found to be low, intraperitoneal (ip) injections can be given at biweekly intervals. For ip  
30 injections, prepare 10-20  $\mu$ gs protein per mouse in a volume of 200-400  $\mu$ ls of PBS per mouse. Using a 1 cc syringe and a 26 gauge needle, inject the solution into the mouse. Do a second tail bleed 10-14 days following injection, and retest the mouse serum titer.

**Example 42: Mouse Serum Titer ELISA**

35 Coat the ELISA plate with 50  $\mu$ l/well of purified antigen at 2  $\mu$ gs/ml PBS. Cover the ELISA plate with parafilm and incubate at 4°C overnight in a humid chamber. Following incubation, wash the plate four times with 200  $\mu$ l/well of PBS per wash.

Block with 3% BSA, 200  $\mu$ ls/well for 60 minutes at room temperature. Shake out blocking solution.

Add serum samples in duplicate, 50  $\mu$ ls/well, at dilutions of  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ , and  $10^{-7}$ , diluted in PBS containing 0.1% BSA. Include blanks of buffer as well as positive and negative control serum at the above dilutions. Incubate at room temp for 1-2 hours. Wash with PBST (PBS with 0.05% tween), 250  $\mu$ ls/well, four times.

Add 50  $\mu$ ls/well of Biotinylated Anti-Mouse IgG at a concentration of 0.5  $\mu$ g/ml in PBST containing 0.1% BSA and 2% Horse Serum. Incubate at room temperature for 30 to 60 minutes. Wash plate four times with PBST.

Add 50  $\mu$ ls/well of ABC reagent (Vector Cat. No. PK-6100) to the plate and incubate at room temperature for 30 minutes. Wash plate six times with PBST.

Prepare substrate for ELISA detection by dissolving 1 tetramethylbenzidine Dihydrochloride (TMB) tablet (Sigma Cat. No. T-3405) in 5 mls. of ddH<sub>2</sub>O. Add 5 mls. of 0.1M Phosphate Citrate Buffer (25.7 mls of 0.2M dibasic sodium phosphate, 24.3 mls of 0.1M citric acid monohydrate, pH 5.0). Add 2  $\mu$ ls of fresh 30% hydrogen peroxide, vortex and use immediately.

Following incubation and washing of the plate, add 100  $\mu$ ls of substrate solution and incubate at room temperature for approximately 15-30 minutes. Stop the reaction by adding 25  $\mu$ ls/well of 2M H<sub>2</sub>SO<sub>4</sub>, and read the plate at 450 nm within 30 minutes versus the controls.

#### **Example 43: Fusion Protocol for Hybridoma Production**

One week prior to the fusion step, make P3X growth medium (1X DMEM 0% (Gibco Cat. No. 11965-019), 5-10% Fetal Bovine Serum, 1X L-Glutamine (Biofluids Cat. No. 300), and 1X Sodium Pyruvate (Biofluids Cat. No. 333). Thaw a new vial of P3X mouse myeloma cells into 1 well of a 6-well dish (see thawing protocol, *infra*) and start expanding them in P3X growth medium. If viability is good the next day transfer to a 100mm dish. Cell density must not exceed  $10^6$  cells/ml or greater. Furthermore, membranes of these cells should not look granular. By the day of the fusion procedure there should 6-8 plates at  $5-8 \times 10^5$  cells/ml. It is a good idea to test some P3X cells in HAT medium. All cells should be dead within around 4 days. If not then P3X cells should be grown in P3X medium containing 15 $\mu$ g/ml 8-azaguanine to eliminate revertants.

Four days prior to the fusion procedure, the mouse should be immunized with an ip injection of approximately 10  $\mu$ g of high purity protein.

One day before the fusion, split the P3X cells and feed them with fresh medium as needed so that cells will be healthy and growing in log phase by the next day.

On the day of the fusion procedure, place 50 mls of P3X media, PEG solution and HAT media (1X DMEM 0%, 20% Fetal Bovine Serum, 4% Hybridoma Supplement-  
5 BM Condensed HI (Boehringer-Mannheim), 1X L-Glutamine, 1X NEAA, 1X Sodium Pyruvate, 1X HAT (Sigma Cat. No. H0262), 1X 0.05M 2ME, and 1X Penicillin-Streptomycin) in a 37°C water bath. Have available approximately 100ml cold DMEM 0%.

Check all P3X plates for possible contamination and to assess health of cells.  
10 Resuspend cells from 4 plates or flasks and combine in 50 ml tubes. Centrifuge at 200 x G for 10 min. Aspirate the supernatant. Resuspend each tube with 10 mls DMEM 0% and pool. Count live cells using trypan blue viability stain (viability should be greater than 90%). The total number of cells should be  $2-4 \times 10^7$  cells. If there are not enough cells then repeat the process with some more plates. Let cells sit at ambient room  
15 temperature (ART) until needed in future step.

Prepare hood where spleen will be removed with: 70% EtOH, sterile instruments including sieve and plunger, 2 petri dishes containing 10 ml DMEM 0% and 15 ml centrifuge tubes (2).

The mouse is sacrificed, and the spleen is then harvested from the carcass. Place  
20 the spleen in the petri dish containing DMEM 0%. Place the sieve in the other dish containing 10 ml DMEM 0% and cover with plate lid. Transfer the spleen to the sieve using a sterile pair of forceps, and using the syringes with needles, tease the spleen apart so that cells spill out into the media. Then, using the other plunger, gently squish the spleen through the sieve. Avoid grinding the spleen organ tissue through the sieve as this  
25 will result in heavy fibroblast growth.

Remove the sieve and transfer the spleen cell suspension to a 15ml centrifuge tube. Wash remaining cells from the dish with 5 mls DMEM 0%, and add to the tube. Allow the tube to sit for 5 minutes to allow large debris to settle to the bottom. Then transfer the cell suspension, minus debris, to the second 15 ml tube. Centrifuge the cells  
30 for 10 min. at 200 x G. Aspirate s/n and resuspend the spleen cells in 5mls DMEM 0%. Add 5 more mls of DMEM 0%, and transfer the entire volume to a 50 ml tube.

Remove 10  $\mu$ ls of the spleen cell suspension, and add to 500  $\mu$ ls of Trypan blue in order to count lymphocytes. (Note: normally a spleen will consistently yield  $10^8$  lymphocytes).

### **Fusion**

To the 50 ml centrifuge tube containing the spleen cells add sufficient P3X cells to make a lymphocyte to P3X cells ratio of 5:1. (e.g. for  $10^8$  lymphocytes you will need  $2 \times 10^7$  P3X cells). Bring the total volume up to 45-50mls with DMEM 0%, and centrifuge at 200 x G for 10 minutes. Prepare a transfer hood with a timer, warm PEG, warm P3X media, and a beaker of water approximately 38-40°C. Aspirate all of the supernatant from the P3X-lymphocyte pellet and attempt to loosen the pellet by flicking the tube. Place the tube in the small water bath. Keep the fusion tube in the warm water, and while gently shaking, add 1 ml of PEG dropwise over 1 minute. Then, let sit with occasional shaking for 1-2 minutes, following which add 1 ml of P3X media dropwise over 1 minute. Next, add 3 mls. of P3X media dropwise over 1 minute, followed by the addition of 10 mls. of P3X media dropwise over 1 minute.

Gently add P3X media to make the total volume 45 mls. Allow the tube to sit for 10 minutes, then centrifuge at 200 x G for 10 minutes. Aspirate the supernatant and gently resuspend the pellet in 5 mls or less of HAT medium. Transfer the cell suspension to the bottle containing 400 mls of HAT medium and swirl to mix. Pour some of the cell suspension into a sterile reservoir.

Plant cells in 96 well plates, 200 $\mu$ ls/well, using a 12 channel pipettor with filtered tips. Place plates in the incubator. Monitor plates every day for hybridoma growth or contamination. Allow plates to incubate for three days. The first feeding (medium change) is done by around day 7 by aspirating off approximately half the media in each well using the 8-position manifold and replacing it with 100-150  $\mu$ ls/well HT medium. Feeding a week or so before the first screening helps to dilute out any antibody produced by the unfused lymphocyte cells which have been found to continue producing antibody after 2 weeks in culture. Many or all of the wells will be ready to be sampled for screening within 2 weeks after the fusion when the colony or colonies fill more than half the well and the supernatant has changed color to a orange/yellow.

### **Example 44: ELISA Screening of Mouse Hybridomas**

To screen the mouse hybridomas, coat the ELISA plate (Immulon 2 "U" bottom microtiter plate (Dynatech Cat. No. 011-010-3555)) with 50  $\mu$ ls/well of the antigen at 2  $\mu$ g/ml PBS. Cover the ELISA plate with plastic seal and incubate at 4°C overnight. Following incubation, wash the plate four times with 200  $\mu$ ls/well of PBS per wash. Block with 3% BSA, 200  $\mu$ ls/well for 60 minutes at room temperature. Shake out blocking solution.

Add hybridoma supernatants, 150  $\mu$ ls/well, into a Corning 96 well assay plate, then transfer 50  $\mu$ ls of each supernatant from the Corning assay plate into the ELISA plate. Include blanks of culture medium as well as positive and negative mouse serum controls. Incubate at room temp for 1-2 hours, or overnight at 4°C. Wash with PBST (PBS with 0.05% tween), 250  $\mu$ ls/well, four times.

Add 50  $\mu$ ls/well of Biotinylated Anti-Mouse IgG H+L, at a concentration of 0.5  $\mu$ g/ml in PBST containing 0.1-0.3% BSA and 1% Horse Serum. Incubate at room temperature for 30 to 60 minutes. Wash plate four times with PBST.

Add 50  $\mu$ ls/well of ABC reagent (Vector Cat. No. PK-6100) to the plate and incubate at room temperature for 30 minutes. Wash plate six times with PBST.

Prepare substrate for ELISA detection by dissolving 1 tetramethylbenzidine Dihydrochloride (TMB) tablet (Sigma Cat. No. T-3405) in 5 mls. of ddH<sub>2</sub>O. Add 5 mls. of 0.1M Phosphate Citrate Buffer (25.7 mls of 0.2M dibasic sodium phosphate, 24.3 mls of 0.1M citric acid monohydrate, pH 5.0). Add 2  $\mu$ ls of fresh 30% hydrogen peroxide, vortex and use immediately.

Following incubation and washing of the plate, add 100  $\mu$ ls of substrate solution and incubate at room temperature for approximately 15-30 minutes. Stop the reaction by adding 25  $\mu$ ls/well of 2M H<sub>2</sub>SO<sub>4</sub>, and read the plate at 450 nm within 30 minutes versus the controls.

#### **Example 45: Testing Relative Affinity of Monoclonals Derived from Culture Supernatants**

##### **A. Determining Antigen Coating Concentration**

Make approximately 1 ml of the antigen at a concentration of 4 ugs/ml in PBS. Transfer to a microdilution tube. Place 0.5 ml PBS in each of 9 microdilution tubes, then do serial dilutions of 1/2 by transferring 0.5 ml from tube to tube starting from the 4 ugs/ml tube. You will now have tubes containing 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.015 and 0.0075 ugs/ml. Coat a plate with the above concentrations, 6 wells each, 50  $\mu$ l/well.

Cover and incubate over night at 4°C. Following incubation, wash the plate four times with 200  $\mu$ ls/well of PBS per wash. Block with 3% BSA, 200  $\mu$ ls/well for 60 minutes at room temperature. Shake out blocking solution.

Look at the titer curve of mouse serum which is positive to the antigen. Determine the serum dilution which is just at the top of the titration curve. Add the positive mouse serum at this dilution in PBS containing 0.1% BSA, 50 $\mu$ ls/well, rows B-D, columns 2-11. Include negative control serum at the above dilution in rows E-G,



columns 2-11. Incubate overnight at 4°C. Following incubation, subtract Negative Control Serum values from Positive Control Serum values. Plot mean value (O.D. 450) against antigen concentration on linear scale. Determine antigen coating concentration which will give a submaximal O.D. This is the coating concentration to use for the relative affinity assay.

**B. Determining the mouse IgG concentration of the hybridoma supernatant sample using the Boehringer Mannheim Biochemica Kit "Mouse-IgG ELISA" (Cat. No. 1333 151)**

Dilute the coating buffer concentrate 1/10 with ddH<sub>2</sub>O. Ten to twenty mls. will be necessary. Obtain an aliquot of capture antibody. Thaw 3 tubes of Post Coating Buffer Concentrate (blocking solution). Twelve wells will be necessary for the standards and 4-6 wells for each supernatant to be tested. Calculate the number of mls of diluted Capture Antibody necessary assuming 50 uls/well of coating volume. Dilute Capture Antibody in the following proportion:

$$\frac{25\text{ul Capture Ab}}{1\text{ ml Coating Buff}} = \frac{X\text{ ul Capture Ab}}{\# \text{ ml Coating Buff}}$$

Coat Nunc plate with the solution and incubate 30 mins at room temperature on a shaker. Dilute the concentrated Post Coating Buffer 1/10 in ddH<sub>2</sub>O. Wash the plate with ELISA wash buffer (0.9% NaCl, 0.1% Tween 20), and block with 200 uls/well of Post Coating Buffer (Block solution) for 15 minutes at room temperature. Dilute the IgG standard into Post Coating Buffer (blocking solution) to the following concentrations: 0.2, 0.1, 0.05, 0.025, 0.0125 and 0.00625 ugs/ml in Post Coating Buffer.

Dilute supernatants into blocking buffer to make final concentrations of 1/100 and 1/1000. Following the blocking step, wash the plate and add 50 uls/well of diluted IgG standards and diluted supernatants in duplicate. Incubate for 30 minutes at room temperature on a shaker.

Dilute the conjugate solution into Post Coating Buffer (block solution) according to the proportion below:

$$\frac{50\text{ul Conjugate}}{1\text{ ml Block sol}} = \frac{X\text{ ul Conjugate}}{\# \text{ ml Block sol}}$$

Wash the plate and add 50ul/well of conjugate. Incubate the plate for 30 minutes at room temperature on shaker.

Dissolve 1 substrate tablet in 5 mls substrate buffer. Wash the plate and add 50  
5 405 nm. uls/well of substrate. Incubate 30 minutes at room temperature on a shaker and read at

### C. Relative Affinity Assay

Coat appropriate ELISA plate(s) overnight at 4°C with the antigen concentration previously determined. Block plate as above. Make 1/3 serial dilutions into PBS + 0.1% BSA of test supernatant.

10 Add 50 uls/well of the dilutions of the supernatant sample, including the undiluted sample, to the ELISA plate(s) in duplicates or triplicates. The positive control consists of a few wells of the positive control mouse serum at the same concentration as used for determining the antigen coating concentration. The negative control consists of a few wells of the dilution buffer. Cover and incubate overnight at 4°C.

15 Plot the IgG concentration of each supernatant against the mean-value (O.D. 450) on a 4 parameter curve fit. Supernatant curves that are more to the left are the supernatants with the highest affinities.

### Example 46: Ascites Production in Mice

20 Hybridoma cells should be healthy and in log phase of growth for ascites production. Transfer cells to a 15 ml. tube and count. Determine the volume which contains  $4 \times 10^6$  cells, transfer that volume to a second tube and centrifuge the cells. Resuspend the pellet in 0.9 mls of HBSS (Hank's Balanced Salt Solution) and transfer to an eppendorf tube.

25 Fill a 1 cc syringe with the cell suspension and inject mice ip as follows: 0.2 cc per mouse if the original cell number was  $4 \times 10^6$  and 0.3 cc if the original cell number was  $3 \times 10^6$ . When the abdomen is very distended and slightly taught to the touch, like a balloon, (usually by day 9 or 10 but sometimes as late as day 14) then it is time to "tap the mouse".

### 30 A. Tapping:

Hold the mouse in your left hand and use an alcohol pad to wipe off the area of the abdomen just above the mouse's left hind leg. While holding the mouse above an open 15 ml centrifuge tube, insert a 19 guage needle into the abdomen. Ascites fluid should immediately begin to drip out of the end of the needle into the centrifuge tube. An  
35 average mouse should yield 3-6 mls. of ascites fluid.

**B. Processing and Storage of Ascites:**

Pool ascitic fluid collected from each mouse in the group (all injected with the same hybridoma) and leave at room temperature for 1-2 hours or place at 37°C for 15-30 minutes. Then place ascites at 4°C overnight to allow for clot formation. Centrifuge  
5 clotted ascites for 10 minutes. Transfer the liquid ascites to a 50 ml centrifuge tube, and store the tube at -20°C. Subsequent taps may be added to this 50 ml tube. When all mice are sacrificed, the pooled ascites can be thawed, respun, and aliquotted for long term storage at -20°C or -70°C. Ascites should be titered by ELISA.

**10 Example 47: Protocol for Freezing and Thawing Mouse Hybridoma and Myeloma Cells****A. Freezing**

Cells to be frozen down should be healthy, in log phase of growth and at a concentration of roughly  $5-8 \times 10^5$  cells/ml. Resuspend cells from a 6 well plate or flask,  
15 transfer to a 15 ml tube and count. Calculate the number of total cells and divide by  $1-3 \times 10^6$  cells per vial to determine the number of vials to be frozen down.

Pellet the cells at 200-300 x G for 5-10 minutes. Aspirate the supernatant from the pellet and resuspend in sufficient cold freeze medium (50% FBS, 10% DMSO in DMEM; or Origen DMSO Freeze Medium (IGEN), Fisher Cat. No. IG-50-0715) to achieve the  
20 desired number of cells/vial per ml (cell densities should be in a range from  $5 \times 10^5$  to  $1 \times 10^7$  cells/vial). Immediately transfer the cell suspension to the cryovials, 1 ml per vial, and place on ice. Transfer the vials to a controlled rate freezer and place the freezer at -70°C for overnight. After 24 hours transfer the vials to a liquid nitrogen tank or -130°C freezer for long term storage.

**25 B. Thawing**

Add 10 ml cold media (e.g. P3X media) to 15 ml tube. Retrieve cryovial of frozen cells and keep on dry ice until ready to thaw. Thaw cells quickly in 37°C water bath. Hold vial during thawing and keep shaking gently until there is just a small bit of ice left in vial. Don't allow the contents to warm above 4°C. Alcohol off the outside of  
30 the cryovial.

Using a sterile pasteur pipette and without touching the edges of the cryovial, transfer the cell suspension in the vial to the 10 mls. of cold media. Spin at 200-300 x G for 5-10 minutes. Aspirate the supernatant and resuspend the pellet in 6 mls. of HT Cloning Media (*supra*). Transfer to 1 well of a 6 well dish. Assess the viability after 24 hours.  
35 Viability should not be less than 50%.

**Example 48: In Vitro Assay for Angiogenic Protein Activity**

The following assay is designed to detect angiogenic protein activity, preferably VEGF-2 activity. For example, a chimeric receptor is generated by fusing the nucleotides encoding for the extracellular domain of the Flt-4 receptor (SEQ ID NO: 27) (Galland *et al.*, *Genomics* 13 (2):475-478 (1992), which is herein incorporated by reference in its entirety), to the nucleotides encoding for the transmembrane domain and intracellular domain of Flk-1 (SEQ ID NO: 28) (Davis-Smith *et al.*, *EMBO J* 15(18):4919-4927 (1996), which is herein incorporated by reference in its entirety). Thus, the chimeric receptor would include amino acids 1 to 775 of SEQ ID NO: 27, fused to amino acids 765 to 1356 of SEQ ID NO: 28, respectively.

Alternatively, the chimeric receptor may be designed as outlined above, but would substitute the transmembrane and intracellular domains of the erythropoietin receptor (EPOR) for the transmembrane and intracellular domains of the Flk-1 receptor, as discussed in Pacifici *et al.*, *JBC* 269(3): 1571-1574 (1994), which is herein incorporated by reference in its entirety (see specifically Figure 1).

The resulting DNA encoding for the chimeric receptor is cloned into an appropriate mammalian, baculoviral, or bacterial expression vector, such as, for example, pC4, pCDNA3, or pA2, as discussed *supra*. Mammalian host cells that could be used for expression of the chimeric receptor include NIH3T3 (*supra*), or the pre-B cell line BaF3 (Achen *et al.*, *PNAS* 95(2): 548-553 (1998), which is herein incorporated by reference in its entirety).

To test for activity, the angiogenic protein can be brought into contact with a cell line expressing the chimeric receptor, or extracts thereof. Then, angiogenic protein binding to the chimeric receptor can be detected by measuring any resulting signal transduced by the chimeric receptor.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of each document cited (including patents, patent applications, journal articles, abstracts, laboratory manuals, books, or other disclosures) in the Background of the Invention, Detailed Description, and Examples is hereby incorporated herein by reference. Moreover, the sequence listing is herein incorporated by reference. Additionally, U.S. Application Serial No. 08/207,412, now issued U.S. Patent No. 5,817,485, and 08/803,926 are hereby incorporated by reference in its entirety.

Applicant's or agent's file reference number	PF112PCT5	International Application No.	UNASSIGNED
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>9</u> , line <u>N/A</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 10801 University Boulevard Manassas, Virginia 20110-2209 United States of America	
Date of deposit 12 May 1995	Accession Number 97149
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
Europe In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28 (4) EPC). Continued on the Attached Pages 2 & 3	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	

For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer <i>Marilyn Younger</i> 6-26-00	Authorized officer

**ATCC Deposit No. 97149****Page N . 2****CANADA**

The applicant requests that, until either a Canadian patent has been issued on the basis of an application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the Commissioner of Patents only authorizes the furnishing of a sample of the deposited biological material referred to in the application to an independent expert nominated by the Commissioner, the applicant must, by a written statement, inform the International Bureau accordingly before completion of technical preparations for publication of the international application.

**NORWAY**

The applicant hereby requests that the application has been laid open to public inspection (by the Norwegian Patent Office), or has been finally decided upon by the Norwegian Patent Office without having been laid open inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Norwegian Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Norwegian Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on the list of recognized experts drawn up by the Norwegian Patent Office or any person approved by the applicant in the individual case.

**AUSTRALIA**

The applicant hereby gives notice that the furnishing of a sample of a microorganism shall only be effected prior to the grant of a patent, or prior to the lapsing, refusal or withdrawal of the application, to a person who is a skilled addressee without an interest in the invention (Regulation 3.25(3) of the Australian Patents Regulations).

**FINLAND**

The applicant hereby requests that, until the application has been laid open to public inspection (by the National Board of Patents and Regulations), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

**UNITED KINGDOM**

The applicant hereby requests that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for the international publication of the application.

**ATCC Dep sit N .: 97149**  
**Page No. 3**

#### **DENMARK**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Danish Patent Office), or has been finally decided upon by the Danish Patent office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Danish Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Danish Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Danish Patent Office or any person by the applicant in the individual case.

#### **SWEDEN**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Swedish Patent Office), or has been finally decided upon by the Swedish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Swedish Patent Office or any person approved by a applicant in the individual case.

#### **NETHERLANDS**

The applicant hereby requests that until the date of a grant of a Netherlands patent or until the date on which the application is refused or withdrawn or lapsed, the microorganism shall be made available as provided in the 31F(1) of the Patent Rules only by the issue of a sample to an expert. The request to this effect must be furnished by the applicant with the Netherlands Industrial Property Office before the date on which the application is made available to the public under Section 22C or Section 25 of the Patents Act of the Kingdom of the Netherlands, whichever of the two dates occurs earlier.

Applicant's or agent's file reference number	PF112PCT5	International application No.	UNASSIGNED
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>9</u> , line <u>NA</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 10801 University Boulevard Manassas, Virginia 20110-2209 United States of America	
Date of deposit 04 March 1994	Accession Number 75698
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
Europe In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28 (4) EPC). Continued on the Attached Pages 2 & 3	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer <i>Marion Younger</i> 6-26-00	Authorized officer



**ATCC Deposit N . 75698****Page No. 2****CANADA**

The applicant requests that, until either a Canadian patent has been issued on the basis of an application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the Commissioner of Patents only authorizes the furnishing of a sample of the deposited biological material referred to in the application to an independent expert nominated by the Commissioner, the applicant must, by a written statement, inform the International Bureau accordingly before completion of technical preparations for publication of the international application.

**NORWAY**

The applicant hereby requests that the application has been laid open to public inspection (by the Norwegian Patent Office), or has been finally decided upon by the Norwegian Patent Office without having been laid open inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Norwegian Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Norwegian Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on the list of recognized experts drawn up by the Norwegian Patent Office or any person approved by the applicant in the individual case.

**AUSTRALIA**

The applicant hereby gives notice that the furnishing of a sample of a microorganism shall only be effected prior to the grant of a patent, or prior to the lapsing, refusal or withdrawal of the application, to a person who is a skilled addressee without an interest in the invention (Regulation 3.25(3) of the Australian Patents Regulations).

**FINLAND**

The applicant hereby requests that, until the application has been laid open to public inspection (by the National Board of Patents and Regulations), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

**UNITED KINGDOM**

The applicant hereby requests that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for the international publication of the application.

**ATCC Deposit No.: 75698****Page No. 3****DENMARK**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Danish Patent Office), or has been finally decided upon by the Danish Patent office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Danish Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Danish Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Danish Patent Office or any person by the applicant in the individual case.

**SWEDEN**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Swedish Patent Office), or has been finally decided upon by the Swedish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Swedish Patent Office or any person approved by a applicant in the individual case.

**NETHERLANDS**

The applicant hereby requests that until the date of a grant of a Netherlands patent or until the date on which the application is refused or withdrawn or lapsed, the microorganism shall be made available as provided in the 31F(1) of the Patent Rules only by the issue of a sample to an expert. The request to this effect must be furnished by the applicant with the Netherlands Industrial Property Office before the date on which the application is made available to the public under Section 22C or Section 25 of the Patents Act of the Kingdom of the Netherlands, whichever of the two dates occurs earlier.

***What Is Claimed Is:***

1. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:
- (a) a polynucleotide fragment of SEQ ID NO:X, which is hybridizable to SEQ ID NO:X;
  - (b) a polynucleotide encoding a polypeptide fragment of SEQ ID NO:Y, which is hybridizable to SEQ ID NO:X;
  - (c) a polynucleotide encoding a polypeptide domain of SEQ ID NO:Y, which is hybridizable to SEQ ID NO:X;
  - (d) a polynucleotide encoding a polypeptide epitope of SEQ ID NO:Y, which is hybridizable to SEQ ID NO:X;
  - (e) a polynucleotide encoding a polypeptide of SEQ ID NO:Y, which is hybridizable to SEQ ID NO:X, having biological activity;
  - (f) a polynucleotide which is a variant of SEQ ID NO:X;
  - (g) a polynucleotide which is an allelic variant of SEQ ID NO:X;
  - (h) a polynucleotide which encodes a species homologue of the SEQ ID NO:Y;
  - (i) a polynucleotide capable of hybridizing under stringent conditions to any one of the polynucleotides specified in (a)-(h), wherein said polynucleotide does not hybridize under stringent conditions to a nucleic acid molecule having a nucleotide sequence of only A residues or of only T residues.
2. The isolated nucleic acid molecule of claim 1, wherein the polynucleotide fragment comprises a nucleotide sequence encoding a secreted protein.
3. The isolated nucleic acid molecule of claim 1, wherein the polynucleotide fragment comprises a nucleotide sequence encoding the sequence identified as SEQ ID NO:Y, which is hybridizable to SEQ ID NO:X.
4. The isolated nucleic acid molecule of claim 1, wherein the polynucleotide fragment comprises the entire nucleotide sequence of SEQ ID NO:X, which is hybridizable to SEQ ID NO:X.
5. The isolated nucleic acid molecule of claim 2, wherein the nucleotide sequence comprises sequential nucleotide deletions from either the C-terminus or the N-terminus.

6. The isolated nucleic acid molecule of claim 3, wherein the nucleotide sequence comprises sequential nucleotide deletions from either the C-terminus or the N-terminus.

7. A recombinant vector comprising the isolated nucleic acid molecule of claim 1.

8. A method of making a recombinant host cell comprising the isolated nucleic acid molecule of claim 1.

9. A recombinant host cell produced by the method of claim 8.

10. The recombinant host cell of claim 9 comprising vector sequences.

11. An isolated polypeptide comprising an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

- (a) a polypeptide fragment of SEQ ID NO:Y;
- (b) a polypeptide fragment of SEQ ID NO:Y, having biological activity;
- (c) a polypeptide domain of SEQ ID NO:Y;
- (d) a polypeptide epitope of SEQ ID NO:Y;
- (e) a secreted form of SEQ ID NO:Y;
- (f) a full length protein of SEQ ID NO:Y;
- (g) a variant of SEQ ID NO:Y;
- (h) an allelic variant of SEQ ID NO:Y; or
- (i) a species homologue of the SEQ ID NO:Y.
- (ii)

12. The isolated polypeptide of claim 11, wherein the secreted form or the full length protein comprises sequential amino acid deletions from either the C-terminus or the N-terminus.

13. An isolated antibody that binds specifically to the isolated polypeptide of claim 11.

14. A recombinant host cell that expresses the isolated polypeptide of claim 11.

15. A method of making an isolated polypeptide comprising:

- (a) culturing the recombinant host cell of claim 14 under conditions such that said polypeptide is expressed; and
- (b) recovering said polypeptide.

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16. The polypeptide produced by claim 15.

17. A method for preventing, treating, or ameliorating a medical condition, comprising administering to a mammalian subject a therapeutically effective amount of the polypeptide of claim 11 or the polynucleotide of claim 1.

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18. A method of diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising:

15

- (a) determining the presence or absence of a mutation in the polynucleotide of claim 1; and
- (b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or absence of said mutation.

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19. A method of diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising:

- (a) determining the presence or amount of expression of the polypeptide of claim 11 in a biological sample; and
- (b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or amount of expression of the polypeptide.

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20. A method for identifying a binding partner to the polypeptide of claim 11 comprising:

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- (a) contacting the polypeptide of claim 11 with a binding partner; and
- (b) determining whether the binding partner effects an activity of the polypeptide.

21. The gene corresponding to the cDNA sequence of SEQ ID NO:Y.

22. A method of identifying an activity in a biological assay, wherein the method comprises:

35

- (a) expressing SEQ ID NO:X in a cell;
- (b) isolating the supernatant;
- (c) detecting an activity in a biological assay; and

200

(d) identifying the protein in the supernatant having the activity.

23. The product produced by the method of claim 20.

5

## FIG. 1A

```

1  GTCCCTCCACCATGCACCTCGCTGGGCTTCTTCTCTGTGGCGTGTCTCTGCTCGCCGCTG 60
   -----+-----+-----+-----+-----+-----+-----+-----+
   CAGGAAGGTGGTACGTGAGCGACCCGGAAGAAGACACCCGACCAAGAGACGAGCGGGGAC
       M H S L G F F S V A C S L L A A A -
   C

61  CGCTGCTCCCGGTCTCGGAGGGCCCGCCGCCGCCGCTTCGAGTCCGGACTCG 120
   -----+-----+-----+-----+-----+-----+-----+-----+
   GCGACGAGGGCCAGGAGCGCTCCGGGGCGGGCGGGCGGGAAGCTCAGGCCCTGAGC
       L L P G P R E A P A A A A A F E S G L D -
   C

121 ACCTCTCGGACGGGAGCCCGACGGGGCGAGGCCACGGCTTATGCAAGCAAGATCTGG 180
   -----+-----+-----+-----+-----+-----+-----+-----+
   TGGAGAGCCTGCGCCTCGGGCTCGGCCCGCTCCGGTGCCGAATACGTCGTTTCTAGACC
       L S D A E P D A G E A T A Y A S K D L E -
   C

181 AGGAGCAGTTACGGTCTGTGCCAGTGTAGATGAACATCATGACTGTACTCTACCCAGAAT 240
   -----+-----+-----+-----+-----+-----+-----+-----+
   TCCTCGTCAATGCCAGACACAGGTCACATCTACTGTAGTACTGACATGAGATGGGTCTTA
       E Q L R S V S S V D E L M T V L Y P E Y -
   C

241 ATTGGAAATGTACAAGTGTACGCTAAGGAAAGGAGGCTGGCAACATAACAGAGAACAGG 300
   -----+-----+-----+-----+-----+-----+-----+-----+
   TAACCTTTTACATGTTACAGTCGATTCTTCTTCCCTCCGACCGTTGTATTGTCTCTTGTC
       W K M Y K C Q L R K G G W Q H N R E Q A -
   C

   CCAACCTCAACTCAAGGACAGAGAGACTATAAATTGCTGCAGCACATTATAATACAG

```

MATCH WITH FIG. 1B





## FIG. 1C

MATCH WITH FIG. 1B

```
661 TTTACAGACAAGTTCATTCCATTATTAGACGTTCCCTGCCAGCAACACTACCACAGTGTC
720
AAATGCTCTGTTCAAGTAAGGTAATAATCTGCAAGGACGGTCGTTGTGATGTTGTCACAG
C Y R Q V H S I I R R S L P A T L P Q C Q
AGGCAGCGAACAAGACCTGCCCCACCAATTACATGTGGAATAATCACATCTGCAGATGCC
721
TCCGTCGCTGTTCTGGACGGGTGTTAATGTACACCTTATTAGTGTAGACGTTCTACGG
C A A N K T C P T N Y M W N N H I C R C L
TGGCTCAGGAAGATTTTATGTTTTCCTCGGATGCTGGAGATGACTCAACAGATGGATTCC
781
ACCGAGTCCTTCTAATAATACAAAAGGAGCCTACGACCTCTACTGAGTTGTCTACCTAAGG
C A Q E D F M F S S D A G D D S T D G F H
ATGACATCTGTGGACCAAAACAAGGAGCTGGATGAAGAGACCTGTCTGCTGTCAGAG
841
TACTGTAGACACCTGGTTGTGTTCCCTCGACCTACTTCTCTGGACAGTCCACACAGCGTCTC
C D I C G P N K E L D E E T C Q C V C R A
CGGGCTCGGCCCTGCCAGCTGTGGACCCCAAGAACTAGACAGAACTCATGCCAGT
901
GCCCCGAAGCCGGACGGTCGACACCTGGGGTGTCTCTGATCTGTCTTTGAGTACGGTCA
C G L R P A S C G P H K E L D R N S C Q C
GTGTCTGTAAAAACAACCTCTTCCCCAGCCAAATGTGGGGCCCAACCGAGAAATTTGATGAAA
961
CACAGACATTTTGTGTTGAGAAAGGGTCCGTTACACCCCGGTTGGCTCTTAAACTACTTT
MATCH WITH FIG. 1D
```

## FIG. 1D

MATCH WITH FIG. 1C

```
C      V C K N K L F P S Q C G A N R E F D E N -
      ACACATGCCAGTGTGTATGTAAGAAGAACCTGCCCCAGAGAAATCAACCCCTAAATCCTGGAA 1080
      TGTGTACGGTCACACATACATTTCTTGGACGGGTCTTTAGTTGGGGATTTAGGACCTT
      T C Q C V C K R T C P R N Q P L N P G K -
      AATGTCCCTGTGAATGTACAGAAAGTCCACAGAAATGCTGTAAAGGAAAGAGTTCC 1140
      TTACACGGACACTTACATGTCTTTCAGGTGTCCTTACGAAACAATTTCTCTTCTTCAAGG
      C A C E C T E S P Q K C L L K G K K F H -
      ACCACCAACATGCAGCTGTTACAGACGGCCCATGTACGAACCCGAGAGGCTGTGTGAGC 1200
      TGGTGGTTTGTACGTCGACAATGTCTGCCGGTACATGCTTGGCGGTCTTCCGAAACACTCG
      H Q T C S C Y R R P C T N R Q K A C E P -
      CAGGATTTTCATATAGTGAAGAAGTGTGTGTCCTTTCATATTGGCAAGACCAC 1260
      GTCCCTAAAAGTATACACTTCTTTCACACAGCAACACAGGGAAGTATAACCGTTTCTGGTG
      G F S Y S E E V C R C V P S Y W Q R P Q -
      AAATGAGCTAAGATTGTACTGTTTCCAGTTCATCGATTTCTATATTATGGAAAACTGTGT
```

MATCH WITH FIG. 1E

MATCH WITH FIG. 1D

[illegible]

U

1 CGAGGCCACGGCTTATGCAAGCAAGATCTGGAGGACAGTTACGGTCTGTGTCCAGTGT  
-----+-----+-----+-----+-----+  
71 AGATGAACTCATGACTGTACTCTACCCAGAATATTGAAATGTACAAGTGTACGCTAAG  
-----+-----+-----+-----+-----+  
M T V L Y P E Y W K M Y K C Q L R  
-----+-----+-----+-----+-----+  
121 GAAAGGAGGCTGGCAACATACAGAGACAGGCCAACCTCAACTCAAGGACAGAAGAGAC  
-----+-----+-----+-----+-----+  
K G G W Q H N R E Q A N L N S R T E E T  
-----+-----+-----+-----+-----+  
181 TATAAAATTGCTGCAGCACATTATAATACAGAGATCTTGAAAAGTATTGATAATGAGTG  
-----+-----+-----+-----+-----+  
I K F A A A H Y N T E I L K S I D N E W  
-----+-----+-----+-----+-----+  
241 GAGAAAGACTCAATGCATGCCACGGGAGGTGTGTATAGATGTGGGAAGGAGTTTGAGT  
-----+-----+-----+-----+-----+  
R K T Q C M P R E V C I D V G K E F G V  
-----+-----+-----+-----+-----+  
301 CGGACAAACACCTTCTTTAAACCTCCATGTGTGTCGGTCTACAGATGTGGGGTTGCTG  
-----+-----+-----+-----+-----+  
A T N T F F K P P C V S V Y R C G G C C  
-----+-----+-----+-----+-----+

FIG. 2A

361 CAATAGTGGGCTGCAGTGCATGAACACACGACGAGCTACCTCAGCAAGACGTTATT  
-----+-----+-----+-----+-----+-----+  
N S E G L Q C M N T S T S Y L S K T L F  
421 TGAATTAACAGTGCCTCTCTCTCAAGGCCCAACACAGTAACAATCAGTTTGGCCAATCA  
-----+-----+-----+-----+-----+-----+  
E I T V P L S Q G P K P V T I S F A N H  
481 CACTTCCTGCCGATGCATGTCTAACTGGATGTTACAGACAAGTTCATTCCATTATTAG  
-----+-----+-----+-----+-----+-----+  
T S C R C M S K L D V Y R Q V H S I I R  
541 ACGTTCCCTGCCAGCAACACTACCACAGTGTGTCAGGCAGCAACAGACCTGCCCCACCAA  
-----+-----+-----+-----+-----+-----+  
R S L P A T L P Q C Q A A N K T C P T N  
601 TTACATGTGGAATAATCACATCTGCAGATGCCCTGGCTCAGGAAGATTTATGTTTTCCTC  
-----+-----+-----+-----+-----+-----+  
Y M W N N H I C R C L A Q E D F M F S S  
661 GGATGCTGGAGATGACTCAACAGATGGATTCCATGACATCTGTGGACCAACAAGGAGCT  
-----+-----+-----+-----+-----+-----+  
D A G D D S T D G F H D I C G P N K E L

FIG. 2B

721 GGATGAAGAGACCTGTCACTGTCTGTCAGAGCGGGCTTCGGCCTGCCAGCTGTGGACC  
-----+-----+-----+-----+-----+  
D E E T C Q C V C R A G L R P A S C G P

781 CCACAAAGAACTAGACAGAACTCATGCCAGTGTGTCTGTATAAAACAACCTCTTCCCCAG  
-----+-----+-----+-----+-----+  
H K E L D R N S C Q C V C K N K L F P S

841 CCAATGTGGGGCCAAACCGAGAAATTTGATGAATAACACATGCCAGTGTGTATGTAAAGAAC  
-----+-----+-----+-----+-----+  
Q C G A N R E F D E N T C Q C V C K R T

901 CTGCCCCAGAAATCAACCCCTAAATCCTGGAAATGTGCCCTGTGAATGTACAGAAAGTCC  
-----+-----+-----+-----+-----+  
C P R N Q P L N P G K C A C E C T E S P

961 ACAGAAATGCTTGTTAAAGGAAGAAGTTCCACCACCAACATGCAGCTGTACAGACG  
-----+-----+-----+-----+-----+  
Q K C L L K G K K F H H Q T C S C Y R R

1021 GCCATGTACGAACCGCCAGAAGGCTTGTGAGCCAGGATTTTCATATAGTGAAGAAGTGTG  
-----+-----+-----+-----+-----+  
P C T N R Q K A C E P G F S Y S E E V C

FIG. 2C

1081 TCGTTGTGTCCTTCATATTTGGCAAAGACCACAAATGAGCTAAGATTGTACTGTTTCCCA  
-----+-----+-----+-----+-----+  
R C V P S Y W Q R P Q M S  
1141 GTTCATCGATTTTCTATTATGGAAAACGTGTGTGCCACAGTAGAACTGTCTGTGAACAGA  
-----+-----+-----+-----+-----+  
1201 GAGACCTTGTGGTCCATGCTAACAAAGACAAAAGTCTGTCTTTCCCTGAACCATGTGGA  
-----+-----+-----+-----+-----+  
1261 TAACTTTACAGAAATGGACTGGAGCTCATCTGC AAAAGGCCCTCTGTAAAGACTGGTTT  
-----+-----+-----+-----+-----+  
1321 CTGCCAATGACCAAAACAGCCCAAGATTTTCCCTCTGTGTGATTTCTTTAAAGAATGACTATA  
-----+-----+-----+-----+-----+  
1381 TAATTTATTTCCACTAAAATAATTGTTTCTGCATTCTTTTATAGCAACAACAAATTGGT  
-----+-----+-----+-----+-----+  
1441 AAACTCACTGTGATCAATATTTTATATCATGC AAAAATATGTTTAAATAAATGAAAA  
-----+-----+-----+-----+-----+  
1501 TTGTATTATATAAAAAAATAAAAAA  
-----+-----+-----+-----+-----+

FIG. 2D

	10	20	30	40																							
1	RTLAC	LLLLGCGYL	LAHVLAEEAE	IPREVI	ERLAR	PDGF-A																					
1	MNR	CWALFLSL	CCYLR	LVSAEGD	PIPEEL	YEMLS	PDGF-B																				
1	PVM	RLEFPC	FLQLL				PIGF																				
1	PVM	RLEFPC	FLQLL				PIGF-2																				
1		NFLLS	WVHW	T			GDGF																				
1		NFLLS	WVHW	S			VEGF																				
1		NFLLS	WVHW	T			VEGF-A																				
1	HSL	GFFSV	ACSL	LAAL	LP	GP	REAP	AAAA	AFES	GL	VEGF-2																
1	SPL			L							VEGF-3																
1									RSSQ	STL	VEGF-D																
1	YRE	WVVVN	VFM	MLY	VQL	VQG	SSNE	HGP	VKR	SSQ	STL	VEGF-D1															
1	KFL	VGIL	VAV	CLH								VEGF-E															
	50	60	70	80																							
36			SQIH	SIRDL	QRLLE	IDS	VG	PDGF-A																			
36			HSIR	SFDDL	QRL	LHG	DPGE	PDGF-B																			
15					AGL	ALPA	VPPQ	PIGF																			
15					AGL	ALPA	VPPQ	PIGF-2																			
12					LALL	YLH	HA	GDGF																			
12					LALL	YLH	HA	VEGF																			
12					LALL	YLH	HA	VEGF-A																			
37	DLSDA	EPDAGE	ATAY	ASKD	LEE	QLRS	VSSV	DEL	MTV	LYPE	VEGF-2																
6								RRLL	LAAL	LQL	VEGF-3																
9	ERS				EQQ	IRA	AASS	LEEL	LRIT	HSE	VEGF-D																
38	ERS				EQQ	IRA	AASS	LEEL	LRIT	HSE	VEGF-D1																
15								QYLL	NAD	STK	VEGF-E																
	90	100	110	120																							
55	S	EDS	LDTS	LRAH	GVHAT	KHVP	EKR	PL	PDGF-A																		
55	E	DGAEL	DLNM	TRSH	SGGE	LES	LARG	RRSL	PDGF-B																		
26	QWAL		SA		GNGS	SEVE	VVP	FQ	PIGF																		
26	QWAL		SA		GNGS	SEVE	VVP	FQ	PIGF-2																		
22	KWSQ		AAPT	TEGE	QKA	HEV	VK	FM	GDGF																		
22	KWSQ		AAPMA	EGGG	QNH	HEV	VK	FM	VEGF																		
22	KWSQ		AAPT	TEGE	QKA	HEV	VK	FM	VEGF-A																		
77	YWKMY	KCQL	RKG	GWQH	NR	EQAN	LNS	RTEE	-TIK	FAAA	HY	VEGF-2															
17	APAQ			APVS	QPD	APGH	QRK	VSWI				VEGF-3															
32	DWKL	WRCR	LRL	KSF		TSM	DSRS	AS	HR	STR	FAAT	FY	VEGF-D														
61	DWKL	WRCR	LRL	KSF		TSM	DSRS	AS	HR	STR	FAAT	FY	VEGF-D1														
25	TWS												VEGF-E														
	130	140	150	160																							
81	PIRRK	RSIE	EEA	VP	AV	C	KTR	T	VI	YE	IP	RS	QVD	PT	SAN	FLI	PDGF-A										
84	G	SLT	IAE	PA	MIA	E	C	KTR	T	EV	FE	IS	RRL	ID	RT	NAN	FLV	PDGF-B									
45			EVW	G	RSY	C	RAL	E	R	L	V	D	V	V	S	BE	YP	SE	VE	H	M	F	S	PIGF			
45			EVW	G	RSY	C	RAL	E	R	L	V	D	V	V	S	BE	YP	SE	VE	H	M	F	S	PIGF-2			
44			DVY	Q	RSY	C	RPI	E	T	L	V	D	I	F	Q	BE	YP	DE	IE	Y	I	F	K	GDGF			
45			DVY	Q	RSY	C	HPI	E	T	L	V	D	I	F	Q	BE	YP	DE	IE	Y	I	F	K	VEGF			
44			DVY	Q	RSY	C	RPI	E	T	L	V	D	I	F	Q	BE	YP	DE	IE	Y	I	F	K	VEGF-A			
115	NTEIL	KSID	NEW	RKT	Q		MPRE	V	C	I	D	V	G	K	E	F	G	V	A	T	N	T	F	F	K	VEGF-2	
40			DVY	T	RAT		QPRE	V	V	P	L	T	V	E	L	M	G	T	V	A	K	Q	L	V	VEGF-3		
66	DIETL	KVIDE	EW	QRT	Q		SPRET	C	VE	V	A	S	E	L	G	K	S	T	N	T	F	F	K	VEGF-D			
95	DIETL	KVIDE	EEW	QRT	Q		SPRET	C	VE	V	A	S	E	L	G	K	S	T	N	T	F	F	K	VEGF-D1			
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Figure 3B

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	170	180	190	200	
120	W P P C V E V K R C T G C C N T S S V K C	Q P S R V H H R S V K V A K V E Y - V	PDGF-A		
121	W P P C V E V Q R C S G C C N N R N V Q C	R P T Q V Q L R P V Q V R K I E I - V	PDGF-B		
75	- P S C V S L L R C T G C C G D E N L H C	V P V E T A N V T M Q L L K I R S G -	PlGF		
75	- P S C V S L L R C T G C C G D E D L H C	V P V E T A N V T M Q L L K I R S G -	PlGF-2		
74	- P S C V P L M R C A G C C N D E A L E C	V P T S E S N V T M Q I M R I K P H -	GDGF		
75	- P S C V P L M R C G C C C N D E G L E C	V P T E S S N I T M Q I M R I K P H -	VEGF		
74	- P S C V P L M R C A G C C N D E A L E C	V P T S E S N V T M Q I M R I K P H -	VEGF-A		
154	- P P C V S V Y R C G G C C N S E G L Q C	M N T S T S Y L S K T L F E I T V P L	VEGF-2		
70	- P S C V T V Q R C G G C C P D D G L E C	V P T G Q H Q V R M Q I L M I R Y P -	VEGF-3		
105	- P P C V N V F R C G G C C N E E S L I C	M N T S T S Y I S K Q L F E I S V P L	VEGF-D		
134	- P P C V N V F R C G G C C N E E S L I C	M N T S T S Y I S K Q L F E I S V P L	VEGF-D1		
58	- P P C V T L M R C G G C C N D E S L E C	V P T E E A N V T M Q L M G A S V S -	VEGF-E		
	210	220	230	240	
159	R K K P K L K E V Q V R L E E H L E C A C	A T T - - - - S L - - - - - N P D	PDGF-A		
160	R K K P I F K K A T V T L E D H L A C K C	E T V - - - - A A A R P V T R S P G	PDGF-B		
113	- D R P S Y - - V E L T F S Q H V R C E C	R P L R E K M K P E R - - - - -	PlGF		
113	- D R P S Y - - V E L T F S Q H V R C E C	R P L R E K M K P E R R R P K G R G K	PlGF-2		
112	- Q S Q H I - - G E M S F L Q H S R C E C	R P K K D R T K P E N - - - - -	GDGF		
113	- Q S Q H I - - G E M S F L Q H N K C E C	R P K K D R A R Q E N - - - - -	VEGF		
112	- Q S Q H I - - G E M S F L Q H S R C E C	R P K K D R T K P E K K S V R G K G K	VEGF-A		
193	S Q G P K P - - V T I S F A N H T S C R C	M S K L D V Y R Q V H S I I R R S L P	VEGF-2		
108	- S S Q - L - - G E M S L E E H S Q C E C	R P K - - - - K K D S A V K P D R A A	VEGF-3		
144	T S V P E L - - V P V K V A N H T G C K C	L P T A P - - R H P Y S I I R R S I Q	VEGF-D		
173	T S V P E L - - V P V K V A N H T G C K C	L P T A P - - R H P Y S I I R R S I Q	VEGF-D1		
96	- G G N G M - - Q H L S F V E H K K C D C	K P P L T T T P P T T T R P P R R - -	VEGF-E		
	250	260	270	280	
188	Y R E E D T G R P R E S - - - - -	- - - - -	- - - - -	- - - - -	PDGF-A
195	G S Q E Q R A K T P Q T R V T I R T V R -	- - - - -	- - - - -	- - - - -	PDGF-B
142	- - - - -	- - - - -	- - - - -	- - - - -	PlGF
150	- - - R R R E K Q R - - - - -	- - - - -	- P T D C -	- - - - -	PlGF-2
141	- - - - -	- - - - -	- H C -	- - - - -	GDGF
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149	G Q K R K R K K S R F K S - - - - -	- W - R V H C -	- - - - -	- - - - -	VEGF-A
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140	T P H H R P Q P R S V P G - - - - -	- W D S A P G -	- - - - -	- - - - -	VEGF-3
180	I P E E D R C S H S K K L C P I D M L W D S N K C K C V L Q E E N P L A G T E D	- - - - -	- - - - -	- - - - -	VEGF-D
209	I P E E D R C S H S K K L C P I D M L W D S N K C K C V L Q E E N P L A G T E D	- - - - -	- - - - -	- - - - -	VEGF-D1
131	- - - - -	- - - - -	- - - - -	- - - - -	VEGF-E
	290	300	310	320	
200	- - - - -	- - - - -	- - - - -	- G K K R K -	PDGF-A
215	- - - - -	- - - - -	- V R R P P K G K H R K -	- - - - -	PDGF-B
142	- - - - -	- - - - -	- - - - -	- C G D - - -	PlGF
161	- - - - -	- - - - -	- - - - -	- H L C G D - - -	PlGF-2
143	- - - - -	- - - - -	- - - - -	- E P C S E R R -	GDGF
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167	- - - - -	- - - - -	- - - - -	- E P C S E R R -	VEGF-A
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131	- - - - -	- - - - -	- - - - -	- - - - -	VEGF-E

Figure 3C

	330	340	350	360	
205	R K R	- - - - -	- - - - -	- - - - -	PDGF-A
226	F K H T	- - - - -	- - - - -	- - - - -	PDGF-B
145	- - - - -	- - - - -	- - - - -	- - - - -	PlGF
166	- - - - -	- - - - -	- - - - -	- - - - -	PlGF-2
150	- - - - -	K H L F	- - - - -	- - - - -	GDGF
151	- - - - -	K H L F	- - - - -	- - - - -	VEGF
174	- - - - -	K H L F	- - - - -	- - - - -	VEGF-A
310	L D R N S C Q C V C K N K L F P S Q C G A N R E F D E N T C Q C V C K R T C P R				VEGF-2
166	- - - - -	T H P T P	- - - - -	- - - - -	VEGF-3
226	- - - - -	P A L C G P H M M F D E D R C E C V C K T P C P K			VEGF-D
255	- - - - -	P A L C G P H M M F D E D R C E C V C K T P C P K			VEGF-D1
131	- - - - -	- - - - -	- - - - -	- - - - -	VEGF-E
	370	380	390	400	
208	- - - - -	- - - - -	- - - - -	- - - - -	PDGF-A
230	- - - - -	- - - - -	H D K T	- - - - -	PDGF-B
145	- - - - -	- - - - -	- - - - -	A V P R	PlGF
166	- - - - -	- - - - -	- - - - -	A V P R	PlGF-2
154	- - V Q D P Q T C K C - S C K N T D S R C K A R Q L E L N E R T C R C D K P R -				GDGF
155	- - V Q D P Q T C K C - S C K N T D S R C K A R Q L E L N E R T C R C D K P R -				VEGF
178	- - V Q D P Q T C K C - S C K N T D S R C K A R Q L E L N E R T C R C D K P R -				VEGF-A
350	N Q P L N P G K C A C - E C T E S P Q K C L L K G K K F H H Q T C S C Y R R P C				VEGF-2
171	- - A P G P S A H A A - P S T T S A L T P G P A A A A A D A A A S S V A K G G -				VEGF-3
251	D L I Q H P K N C S C F E C K E S L E T C C Q K H K L F H P D T C S C E D R - C				VEGF-D
280	D L I Q H P K N C S C F E C K E S L E T C C Q K H K L F H P D T C S C E D R - C				VEGF-D1
131	- - - - -	- - - - -	- - - - -	R -	VEGF-E
	410	420	430		
208	- - - - -	- - - - -	- - - - -	L K P T	PDGF-A
234	- - - - -	- - - - -	- - - - -	A L K E T L G A	PDGF-B
149	- - - - -	- - - - -	- - - - -	- - - - - R	PlGF
170	- - - - -	- - - - -	- - - - -	- - - - - R	PlGF-2
190	- - - - -	- - - - -	- - - - -	- - - - - R	GDGF
191	- - - - -	- - - - -	- - - - -	- - - - - R	VEGF
214	- - - - -	- - - - -	- - - - -	- - - - - R	VEGF-A
389	T N R Q K A C E P G F S Y S E E V C R C V P S Y W - - - - -		Q R P Q M S		VEGF-2
207	- - - - -	- - - - -	- - - - -	- - - - - A	VEGF-3
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319	P F H T R P C A S G K T A C A K H C R F P K E K R A A Q G P H S R K N P				VEGF-D1
132	- - - - -	- - - - -	- - - - -	- - - - - R	VEGF-E

Decoration 'Decoration #1': Shade (with solid black) residues that match the consensus named 'Consensus #1' exactly.

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 <213> Homo sapiens

<400> 6  
 Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala Gly  
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Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser Ala Gly  
 20 25 30

5

Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu Val Trp Gly  
 35 40 45

Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp Val Val Ser Glu  
 50 55 60

Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser Cys Val Ser Leu  
 65 70 75 80

Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asn Leu His Cys Val Pro  
 85 90 95

Val Glu Thr Ala Asn Val Thr Met Gln Leu Leu Lys Ile Arg Ser Gly  
 100 105 110

Asp Arg Pro Ser Tyr Val Glu Leu Thr Phe Ser Gln His Val Arg Cys  
 115 120 125

Glu Cys Arg Pro Leu Arg Glu Lys Met Lys Pro Glu Arg Cys Gly Asp  
 130 135 140

Ala Val Pro Arg Arg  
 145

&lt;210&gt; 7

&lt;211&gt; 597

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 7

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 gaggtggaag tggtaaccctt ccaggaagtg tggggccgca gctactgccg ggcgctggag 180  
 aggctggtgg acgtcgtgtc cgagtacccc agcgagggtg agcacatgtt cagcccatcc 240  
 tgtgtctccc tgctgcgtg caccggctgc tgcggcgatg aggatctgca ctgtgtgccg 300  
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 cccacagact gccacctgtg cggcgatgct gttccccgga ggtaaccac cccttgagg 540  
 agagagaccc cgcacccggc tcgtgtattt attaccgtca cactcttcag tgactcc 597

&lt;210&gt; 8

&lt;211&gt; 170

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 8

Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala Gly  
 1 5 10 15

Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser Ala Gly  
 20 25 30

Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu Val Trp Gly  
 35 40 45

Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp Val Val Val Ser  
 50 55 60

6

Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser Cys Val Ser Leu  
65 70 75 80

Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asp Leu His Cys Val Pro  
85 90 95

Val Glu Thr Ala Asn Val Thr Met Gln Leu Leu Lys Ile Arg Ser Gly  
100 105 110

Asp Arg Pro Ser Tyr Val Glu Leu Thr Phe Ser Gln His Val Arg Cys  
115 120 125

Glu Cys Arg Pro Leu Arg Glu Lys Met Lys Pro Glu Arg Arg Arg Pro  
130 135 140

Lys Gly Arg Gly Lys Arg Arg Arg Glu Lys Gln Arg Pro Thr Asp Cys  
145 150 155 160

His Leu Cys Gly Asp Ala Val Pro Arg Arg  
165 170

&lt;210&gt; 9

&lt;211&gt; 577

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 9

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caacgtcact atgcagatca tgcggatcaa acctcaccaa agccagcaca taggagagat 360  
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&lt;210&gt; 10

&lt;211&gt; 190

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 10

Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu Leu  
1 5 10 15

Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr Glu Gly  
20 25 30

Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val Tyr Gln Arg  
35 40 45

Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr  
50 55 60

Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met  
65 70 75 80



7

Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala Leu Glu Cys Val Pro Thr  
85 90 95

Ser Glu Ser Asn Val Thr Met Gln Ile Met Arg Ile Lys Pro His Gln  
100 105 110

Ser Gln His Ile Gly Glu Met Ser Phe Leu Gln His Ser Arg Cys Glu  
115 120 125

Cys Arg Pro Lys Lys Asp Arg Thr Lys Pro Glu Asn His Cys Glu Pro  
130 135 140

Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln Thr Cys  
145 150 155 160

Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu  
165 170 175

Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg  
180 185 190

<210> 11

<211> 989

**<212> DNA**

<213> Homo sapiens

<400> 11

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gttcatggat	gtctatcagc	gcagctactg	ccatccaatc	gagacctctg	tggacatctt	240
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caccatgcag	attatgcgga	tcaaacctca	ccaaggccag	cacataggag	agatgagctt	420
cctacagcac	aacaaatgtg	aatgcagacc	aaagaagat	agagcaagac	aagaaaatcc	480
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<210> 12

&lt;211&gt; 191

<212> PRT

<213> Homo sapiens

<400> 12

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu  
1 5 10 15

Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly  
20 25 30

Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln  
35 40 45

Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu  
 50 55 60  
 Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu  
 65 70 75 80  
 Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro  
 85 90 95  
 Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His  
 100 105 110  
 Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys  
 115 120 125  
 Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Asn Pro Cys Gly  
 130 135 140  
 Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln Thr  
 145 150 155 160  
 Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln  
 165 170 175  
 Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg  
 180 185 190

<210> 13  
 <211> 649  
 <212> DNA  
 <213> Homo sapiens

<400> 13  
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 ggtgaagttc atggacgtct accagcgcag ctattgccgt ccgattgaga ccctggtgga 180  
 catcttccag gagtaccocg atgagataga gtatatcttc aagccgtcct gtgtgcccct 240  
 aatgcggtgt gcgggctgct gcaatgatga agccctggag tgcgtgcccc cgteggagag 300  
 caacgtcact atgcagatca tgcggatcaa acctcacaa agccagcaca taggagagat 360  
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 taaatcctgg agagttcact gtgagccttg ttcagagcgg agaaagcatt tgtttgtcca 540  
 agatccgcag acgtgtaaat gttcctgcaa aaacacagac tcgcgttgca aggcgaggca 600  
 gcttgagtta aacgaacgta cttgcagatg tgacaagcca aggcgggtga 649

<210> 14  
 <211> 214  
 <212> PRT  
 <213> Homo sapiens

<400> 14  
 Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu Leu  
 1 5 10 15  
 Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr Glu Gly  
 20 25 30  
 Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val Tyr Gln Arg

35 40 45

Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr  
50 55 60

Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met  
65 70 75 80

Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala Leu Glu Cys Val Pro Thr  
85 90 95

Ser Glu Ser Asn Val Thr Met Gln Ile Met Arg Ile Lys Pro His Gln  
100 105 110

Ser Gln His Ile Gly Glu Met Ser Phe Leu Gln His Ser Arg Cys Glu  
115 120 125

Cys Arg Pro Lys Lys Asp Arg Thr Lys Pro Glu Lys Lys Ser Val Arg  
130 135 140

Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Phe Lys  
145 150 155 160

Ser Trp Arg Val His Cys Glu Pro Cys Ser Glu Arg Arg Lys His Leu  
165 170 175

Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp  
180 185 190

Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys Arg  
195 200 205

Cys Asp Lys Pro Arg Arg  
210

<210> 15  
<211> 1674  
<212> DNA  
<213> Homo sapiens

<400> 15

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acctctcgga cgcggagccc gacgcgggag aggccacggc ttatgcaagc aaagatctgg 180  
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ccaacctcaa ctcaaggaca gaagagacta taaaatttgc tgcagcacat tataatacag 360  
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10

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gcaaaatatg tttaaaataa aatgaaaatt gtatttataa aaaaaaaaaa aaaa 1674

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&lt;210&gt; 16

&lt;211&gt; 419

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 16

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Met His Ser Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala
  1           5           10           15

Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Ala Phe
  20           25           30

Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala
  35           40           45

Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser
  50           55           60

Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met
  65           70           75           80

Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln
  85           90           95

Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala
 100           105           110

His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys
 115           120           125

Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe
 130           135           140

Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr
 145           150           155           160

Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr
 165           170           175

Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu
 180           185           190

Ser Gln Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser
 195           200           205

Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile
 210           215           220

Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn
 225           230           235           240

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<210> 17
<211> 624
<212> DNA
<213> Homo sapiens
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<400> 17							
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<210> 18
<211> 207
<212> PRT
<213> Homo sapiens
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&lt;400&gt; 18

Met Ser Pro Leu Leu Arg Arg Leu Leu Leu Ala Ala Leu Leu Gln Leu  
 1 5 10 15

Ala Pro Ala Gln Ala Pro Val Ser Gln Pro Asp Ala Pro Gly His Gln  
 20 25 30

Arg Lys Val Val Ser Trp Ile Asp Val Tyr Thr Arg Ala Thr Cys Gln  
 35 40 45

Pro Arg Glu Val Val Val Pro Leu Thr Val Glu Leu Met Gly Thr Val  
 50 55 60

Ala Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly  
 65 70 75 80

Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln  
 85 90 95

Val Arg Met Gln Ile Leu Met Ile Arg Tyr Pro Ser Ser Gln Leu Gly  
 100 105 110

Glu Met Ser Leu Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys Lys  
 115 120 125

Lys Asp Ser Ala Val Lys Pro Asp Arg Ala Ala Thr Pro His His Arg  
 130 135 140

Pro Gln Pro Arg Ser Val Pro Gly Trp Asp Ser Ala Pro Gly Ala Pro  
 145 150 155 160

Ser Pro Ala Asp Ile Thr His Pro Thr Pro Ala Pro Gly Pro Ser Ala  
 165 170 175

His Ala Ala Pro Ser Thr Thr Ser Ala Leu Thr Pro Gly Pro Ala Ala  
 180 185 190

Ala Ala Ala Asp Ala Ala Ala Ser Ser Val Ala Lys Gly Gly Ala  
 195 200 205

&lt;210&gt; 19

&lt;211&gt; 2846

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 19

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 attgtagctg ctactatttt ggcacatctaa tcttaatatc atttattttg tagtccttgg 660  
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ttttcccagg tgttaaaaaa aaaaaa 2846

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&lt;210&gt; 20

&lt;211&gt; 325

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 20

Met Arg Ser Ser Gln Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg  
1 5 10 15

Ala Ala Ser Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp  
20 25 30

Trp Lys Leu Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met  
35 40 45

Asp Ser Arg Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe  
50 55 60

Tyr Asp Ile Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr  
65 70 75 80

Gln Cys Ser Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly  
85 90 95

14

Lys Ser Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg  
 100 105 110  
 Cys Gly Gly Cys Cys Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser  
 115 120 125  
 Thr Ser Tyr Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr  
 130 135 140  
 Ser Val Pro Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys  
 145 150 155 160  
 Lys Cys Leu Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg  
 165 170 175  
 Ser Ile Gln Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu  
 180 185 190  
 Cys Pro Ile Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu  
 195 200 205  
 Gln Glu Glu Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln  
 210 215 220  
 Glu Pro Ala Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys  
 225 230 235 240  
 Glu Cys Val Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro  
 245 250 255  
 Lys Asn Cys Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys  
 260 265 270  
 Gln Lys His Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg  
 275 280 285  
 Cys Pro Phe His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala  
 290 295 300  
 Lys His Cys Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His  
 305 310 315 320  
 Ser Arg Lys Asn Pro  
 325

&lt;210&gt; 21

&lt;211&gt; 2004

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 21

ccagctttct gtarctgtaa gcattggtgg ccacaccacc tccttacaaa gcaactagaa 60  
 cctgceggcat acattggaga gattttttta attttctgga caygaagtaa atttagagtg 120  
 ctttcyaatt tcaggtagaa gacatgtcca ccttctgatt atttttggag aacattttga 180  
 tttttttcat ctctctctcc ccacccttaa gattgtgcaa aaaaagcgta ccttgccctaa 240  
 ttgaaataat ttcattggat tttgatcaga actgatcatt tggttttctg tgtgaagttt 300  
 tgagggtttca aactttcctt ctggagaatg ccttttgaaa caattttctc tagctgcctg 360  
 atgtcaactg cttagtaatc agtggatatt gaaatattca aaatgtacag agagtgggta 420  
 gtgggtgaatg ttttcatgat gttgtacgtc cagctggtgc agggctccag taatgaacat 480  
 ggaccagtga agcgatcatc tcagtccaca ttggaacgat ctgaacagca gatcagggct 540



15

```

gcttctagtt tggaggaact acttcgaatt actcactctg aggactggaa gctgtggaga 600
tgcaggctga ggctcaaaag ttttaccagt atggactctc gctcagcatc ccacgggtcc 660
actaggtttg cggcaacttt ctatgacatt gaaacactaa aagttataga tgaagaatgg 720
caaagaactc agtgcagccc tagagaaacy tgcgtggagg tggccagtga gctgggggaa 780
agtaccaaca cattcttcaa gcccccttgt gtgaacgtgt tccgatgtgg tggctgttgc 840
aatgaagaga gccttatctg tatgaacacc agcacctcgt acatttccaa acagctcttt 900
gagatatcag tgcctttgac atcagtacct gaattagtgc ctgttaaagt tgccaatcat 960
acaggttgta agtgcttgcc aacagcccc cgccatccat actcaattat cagaagatcc 1020
atccagatcc ctgaagaaga tcgctgttcc cattccaaga aactctgtcc tattgacatg 1080
ctatgggata gcaacaaatg taaatgtgtt ttgcaggagg aaaatccact tgctggaaca 1140
gaagaccact ctcacttcca ggaaccagct ctctgtgggc cacacatgat gtttgacgaa 1200
gatcgttgcg agtgtgtctg taaaacacca tgtcccaaag atctaacca gcaccccaa 1260
aactgcagtg gccttgagtg caaagaaagt ctggagacct gctgccagaa gcacaagcta 1320
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ttaacagcat gctgctttgc caagtgtctg tcaactgttt tttcccaggt gttaaaaaaa 1560
aaatccattt tacacagcac cacagtgaat ccagaccaac cttccattca caccagtaa 1620
ggagtccctg gttcattgat ggatgtcttc tagctgcaga tgccctctgcg caccaaggaa 1680
tggagaggag gggaccatg taatcctttt gtttagtttt gtttttgtt tttggtgaat 1740
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gaggaaaact gtagtctctg agtcctttgc taatcgcaac tcttgatgaat tattctgatt 1860
cttttttatg cagaatttga ttcgtatgat cagtactgac tttctgatta ctgtccagct 1920
tatagtcttc cagtttaatg aactaccatc tgatgtttca tatttaagtg tatttaaaga 1980
aaataaacac cattattcaa gtct 2004

```

&lt;210&gt; 22

&lt;211&gt; 354

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 22

```

Met Tyr Arg Glu Trp Val Val Val Asn Val Phe Met Met Leu Tyr Val
 1             5             10             15

```

```

Gln Leu Val Gln Gly Ser Ser Asn Glu His Gly Pro Val Lys Arg Ser
      20             25             30

```

```

Ser Gln Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser
      35             40             45

```

```

Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp Trp Lys Leu
      50             55             60

```

```

Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp Ser Arg
      65             70             75             80

```

```

Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile
      85             90             95

```

```

Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser
      100            105            110

```

```

Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Ser Thr
      115            120            125

```

```

Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly
      130            135            140

```

```

Cys Cys Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser Tyr

```

16

145                      150                      155                      160  
 Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro  
                                  165                                   170                                   175  
 Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu  
                                  180                                   185                                   190  
 Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln  
                                  195                                   200                                   205  
 Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile  
                                  210                                   215                                   220  
 Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Glu  
                                  225                                   230                                   235                                   240  
 Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala  
                                  245                                   250                                   255  
 Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val  
                                  260                                   265                                   270  
 Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys  
                                  275                                   280                                   285  
 Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His  
                                  290                                   295                                   300  
 Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe  
                                  305                                   310                                   315                                   320  
 His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys  
                                  325                                   330                                   335  
 Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys  
                                  340                                   345                                   350

Asn Pro

<210> 23  
 <211> 399  
 <212> DNA  
 <213> Homo sapiens

<400> 23  
 atgaagtttc tcgtcgccat actggtagct gtgtgcttgc accagtatct gctgaacgcg 60  
 gacagcacga aaacatggtc cgaagtgttt gaaaacagcg ggtgcaagcc aaggccgatg 120  
 gtctttcgag tacacgacga gcacccggag ctaacttctc agcggttcaa cccgccgtgt 180  
 gtcacgttga tgcgatgcgg cgggtgctgc aacgacgaga gcttagaatg cgccccacg 240  
 gaagaggcaa acgtaacgat gcaactcatg ggagcgtcgg tctccggtgg taacgggatg 300  
 caacatctga gtttcgtaga gcataagaaa tgcgattgta aaccaccact cagcaccacg 360  
 ccaccgacga ccacaaggcc gcccaagaaga cgccgctag 399

<210> 24  
 <211> 132  
 <212> PRT  
 <213> Homo sapiens

&lt;400&gt; 24

Met Lys Phe Leu Val Gly Ile Leu Val Ala Val Cys Leu His Gln Tyr  
 1 5 10 15

Leu Leu Asn Ala Asp Ser Thr Lys Thr Trp Ser Glu Val Phe Glu Asn  
 20 25 30

Ser Gly Cys Lys Pro Arg Pro Met Val Phe Arg Val His Asp Glu His  
 35 40 45

Pro Glu Leu Thr Ser Gln Arg Phe Asn Pro Pro Cys Val Thr Leu Met  
 50 55 60

Arg Cys Gly Gly Cys Cys Asn Asp Glu Ser Leu Glu Cys Val Pro Thr  
 65 70 75 80

Glu Glu Ala Asn Val Thr Met Gln Leu Met Gly Ala Ser Val Ser Gly  
 85 90 95

Gly Asn Gly Met Gln His Leu Ser Phe Val Glu His Lys Lys Cys Asp  
 100 105 110

Cys Lys Pro Pro Leu Thr Thr Thr Pro Pro Thr Thr Thr Arg Pro Pro  
 115 120 125

Arg Arg Arg Arg  
 130

&lt;210&gt; 25

&lt;211&gt; 1526

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 25

cgaggccacg gcttatgcaa gcaaagatct ggaggagcag ttacggctctg tgtccagtgt 60  
 agatgaactc atgactgtac tctaccaga atattggaaa atgtacaagt gtcagctaag 120  
 gaaaggaggc tggcaacata acagagaaca ggccaacctc aactcaagga cagaagagac 180  
 tataaaattt gctgcagcac attataatac agagatcttg aaaagtattg ataatgagtg 240  
 gagaaagact caatgcatgc cacgggaggt gtgtatagat gtggggaagg agtttggagt 300  
 cgcgacaaac acctcttcta aacctccatg tgtgtccgtc tacagatgtg ggggttgctg 360  
 caatagttag gggctgcagt gcatgaacac cagcacgagc tacctcagca agacgttatt 420  
 tgaaattaca gtgcctctct ctcaaggccc caaaccagta acaatcagtt ttgccaatca 480  
 cacttcctgc cgatgcatgt ctaaactgga tgtttacaga caagttcatt ccattattag 540  
 acgttccctg ccagcaacac taccacagtg tcaggcagcg aacaagacct gccccacca 600  
 ttacatgtgg aataatcaca tctgcagatg cctggctcag gaagatttta tgttttccctc 660  
 ggatgctgga gatgactcaa cagatggatt ccattgacatc tgtggacca acaaggagct 720  
 ggatgaagag acctgtcagt gtgtctgcag agcggggctt cggcctgcca gctgtggacc 780  
 ccacaaagaa ctagacagaa actcatgcca gtgtgtctgt aaaaacaaac tcttccccag 840  
 ccaatgtggg gccaaccgag aatttgatga aaacacatgc cagtgtgtat gtaaaagaac 900  
 ctgccccaga aatcaacccc taaatcctgg aaaatgtgcc tgtgaatgta cagaaagtcc 960  
 acagaaatgc ttgttaaaag gaaagaagtt ccaccaccaa acatgcagct gttacagacg 1020  
 gccatgtacg aaccgccaag aggcctgtga gccaggattt tcatatagtg aagaagtgtg 1080  
 tcgttgtgtc ccttcattat ggcaaagacc acaaatgagc taagattgta ctgttttcca 1140  
 gttcatcgat tttctattat ggaaaactgt gttgccacag tagaactgtc tgtgaacaga 1200  
 gagacccttg tgggtccatg ctaacaaaga caaaagtctg tctttcctga accatgtgga 1260  
 taactttaca gaaatggact ggagctcatc tgcaaaaggc ctcttgtaaa gactggtttt 1320  
 ctgccaatga ccaaacagcc aagattttcc tcttgtgatt tctttaaaag aatgactata 1380  
 taattttatt ccactaaaaa tattgtttct gcattcattt ttatagcaac aacaattggt 1440  
 aaaactcact gtgatcaata tttttatata atgcaaaata tgtttaaaat aaaatgaaaa 1500

18

ttgtatttat aaaaaaaaaa aaaaaa

1526

&lt;210&gt; 26

&lt;211&gt; 350

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 26

Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Tyr Lys Cys Gln Leu  
 1 5 10 15

Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln Ala Asn Leu Asn Ser  
 20 25 30

Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr Asn Thr Glu  
 35 40 45

Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln Cys Met Pro  
 50 55 60

Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val Ala Thr Asn  
 65 70 75 80

Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys Gly Gly Cys  
 85 90 95

Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr Ser Tyr Leu  
 100 105 110

Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln Gly Pro Lys  
 115 120 125

Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg Cys Met Ser  
 130 135 140

Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg Arg Ser Leu  
 145 150 155 160

Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr Cys Pro Thr  
 165 170 175

Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala Gln Glu Asp  
 180 185 190

Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp Gly Phe His  
 195 200 205

Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys  
 210 215 220

Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro His Lys Glu  
 225 230 235 240

Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe Pro  
 245 250 255

Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys  
 260 265 270

Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys

19

275 280 285

Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu Leu Lys Gly  
290 295 300

Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys Thr  
305 310 315 320

Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser Glu Glu Val  
325 330 335

Cys Arg Cys Val Pro Ser Tyr Trp Gln Arg Pro Gln Met Ser  
340 345 350

&lt;210&gt; 27

&lt;211&gt; 1298

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 27

Met Gln Arg Gly Ala Ala Leu Cys Leu Arg Leu Trp Leu Cys Leu Gly  
1 5 10 15

Leu Leu Asp Gly Leu Val Ser Asp Tyr Ser Met Thr Pro Pro Thr Leu  
20 25 30

Asn Ile Thr Glu Glu Ser His Val Ile Asp Thr Gly Asp Ser Leu Ser  
35 40 45

Ile Ser Cys Arg Gly Gln His Pro Leu Glu Trp Ala Trp Pro Gly Ala  
50 55 60

Gln Glu Ala Pro Ala Thr Gly Asp Lys Asp Ser Glu Asp Thr Gly Val  
65 70 75 80

Val Arg Asp Cys Glu Gly Thr Asp Ala Arg Pro Tyr Cys Lys Val Leu  
85 90 95

Leu Leu His Glu Val His Ala Asn Asp Thr Gly Ser Tyr Val Cys Tyr  
100 105 110

Tyr Lys Tyr Ile Lys Ala Arg Ile Glu Gly Thr Thr Ala Ala Ser Ser  
115 120 125

Tyr Val Phe Val Arg Asp Phe Glu Gln Pro Phe Ile Asn Lys Pro Asp  
130 135 140

Thr Leu Leu Val Asn Arg Lys Asp Ala Met Trp Val Pro Cys Leu Val  
145 150 155 160

Ser Ile Pro Gly Leu Asn Val Thr Leu Arg Ser Gln Ser Ser Val Leu  
165 170 175

Trp Pro Asp Gly Gln Glu Val Val Trp Asp Asp Arg Arg Gly Met Leu  
180 185 190

Val Ser Thr Pro Leu Leu His Asp Ala Leu Tyr Leu Gln Cys Glu Thr  
195 200 205

Thr Trp Gly Asp Gln Asp Phe Leu Ser Asn Pro Phe Leu Val His Ile

20

210	215	220
Thr Gly Asn Glu Leu Tyr Asp Ile Gln Leu Leu Pro Arg Lys Ser Leu		
225	230	235 240
Glu Leu Leu Val Gly Glu Lys Leu Val Leu Asn Cys Thr Val Trp Ala		
	245	250 255
Glu Phe Asn Ser Gly Val Thr Phe Asp Trp Asp Tyr Pro Gly Lys Gln		
	260	265 270
Ala Glu Arg Gly Lys Trp Val Pro Glu Arg Arg Ser Gln Gln Thr His		
	275	280 285
Thr Glu Leu Ser Ser Ile Leu Thr Ile His Asn Val Ser Gln His Asp		
	290	295 300
Leu Gly Ser Tyr Val Cys Lys Ala Asn Asn Gly Ile Gln Arg Phe Arg		
305	310	315 320
Glu Ser Thr Glu Val Ile Val His Glu Asn Pro Phe Ile Ser Val Glu		
	325	330 335
Trp Leu Lys Gly Pro Ile Leu Glu Ala Thr Ala Gly Asp Glu Leu Val		
	340	345 350
Lys Leu Pro Val Lys Leu Ala Ala Tyr Pro Pro Pro Glu Phe Gln Trp		
	355	360 365
Tyr Lys Asp Gly Lys Ala Leu Ser Gly Arg His Ser Pro His Ala Leu		
	370	375 380
Val Leu Lys Glu Val Thr Glu Ala Ser Thr Gly Thr Tyr Thr Leu Ala		
385	390	395 400
Leu Trp Asn Ser Ala Ala Gly Leu Arg Arg Asn Ile Ser Leu Glu Leu		
	405	410 415
Val Val Asn Val Pro Pro Gln Ile His Glu Lys Glu Ala Ser Ser Pro		
	420	425 430
Ser Ile Tyr Ser Arg His Ser Arg Gln Ala Leu Thr Cys Thr Ala Tyr		
	435	440 445
Gly Val Pro Leu Pro Leu Ser Ile Gln Trp His Trp Arg Pro Trp Thr		
	450	455 460
Pro Cys Lys Met Phe Ala Gln Arg Ser Leu Arg Arg Arg Gln Gln Gln		
465	470	475 480
Asp Leu Met Pro Gln Cys Arg Asp Trp Arg Ala Val Thr Thr Gln Asp		
	485	490 495
Ala Val Asn Pro Ile Glu Ser Leu Asp Thr Trp Thr Glu Phe Val Glu		
	500	505 510
Gly Lys Asn Lys Thr Val Ser Lys Leu Val Ile Gln Asn Ala Asn Val		
	515	520 525
Ser Ala Met Tyr Lys Cys Val Val Ser Asn Lys Val Gly Gln Asp Glu		
	530	535 540

Arg Leu Ile Tyr Phe Tyr Val Thr Thr Ile Pro Asp Gly Phe Thr Ile  
 545 550 555 560  
 Glu Ser Lys Pro Ser Glu Glu Leu Leu Glu Gly Gln Pro Val Leu Leu  
 565 570 575  
 Ser Cys Gln Ala Asp Ser Tyr Lys Tyr Glu His Leu Arg Trp Tyr Arg  
 580 585 590  
 Leu Asn Leu Ser Thr Leu His Asp Ala His Gly Asn Pro Leu Leu Leu  
 595 600 605  
 Asp Cys Lys Asn Val His Leu Phe Ala Thr Pro Leu Ala Ala Ser Leu  
 610 615 620  
 Glu Glu Val Ala Pro Gly Ala Arg His Ala Thr Leu Ser Leu Ser Ile  
 625 630 635 640  
 Pro Arg Val Ala Pro Glu His Glu Gly His Tyr Val Cys Glu Val Gln  
 645 650 655  
 Asp Arg Arg Ser His Asp Lys His Cys His Lys Lys Tyr Leu Ser Val  
 660 665 670  
 Gln Ala Leu Glu Ala Pro Arg Leu Thr Gln Asn Leu Thr Asp Leu Leu  
 675 680 685  
 Val Asn Val Ser Asp Ser Leu Glu Met Gln Cys Leu Val Ala Gly Ala  
 690 695 700  
 His Ala Pro Ser Ile Val Trp Tyr Lys Asp Glu Arg Leu Leu Glu Glu  
 705 710 715 720  
 Lys Ser Gly Val Asp Leu Ala Asp Ser Asn Gln Lys Leu Ser Ile Gln  
 725 730 735  
 Arg Val Arg Glu Glu Asp Ala Gly Pro Tyr Leu Cys Ser Val Cys Arg  
 740 745 750  
 Pro Lys Gly Cys Val Asn Ser Ser Ala Ser Val Ala Val Glu Gly Ser  
 755 760 765  
 Glu Asp Lys Gly Ser Met Glu Ile Val Ile Leu Val Gly Thr Gly Val  
 770 775 780  
 Ile Ala Val Phe Phe Trp Val Leu Leu Leu Leu Ile Phe Cys Asn Met  
 785 790 795 800  
 Arg Arg Pro Ala His Ala Asp Ile Lys Thr Gly Tyr Leu Ser Ile Ile  
 805 810 815  
 Met Asp Pro Gly Glu Val Pro Leu Glu Glu Gln Cys Glu Tyr Leu Ser  
 820 825 830  
 Tyr Asp Ala Ser Gln Trp Glu Phe Pro Arg Glu Arg Leu His Leu Gly  
 835 840 845  
 Arg Val Leu Gly Tyr Gly Ala Phe Gly Lys Val Val Glu Ala Ser Ala  
 850 855 860

22

Phe Gly Ile His Lys Gly Ser Ser Cys Asp Thr Val Ala Val Lys Met  
 865 870 875 880  
 Leu Lys Glu Gly Ala Thr Ala Ser Glu Gln Arg Ala Leu Met Ser Glu  
 885 890 895  
 Leu Lys Ile Leu Ile His Ile Gly Asn His Leu Asn Val Val Asn Leu  
 900 905 910  
 Leu Gly Ala Cys Thr Lys Pro Gln Gly Pro Leu Met Val Ile Val Glu  
 915 920 925  
 Phe Cys Lys Tyr Gly Asn Leu Ser Asn Phe Leu Arg Ala Lys Arg Asp  
 930 935 940  
 Ala Phe Ser Pro Cys Ala Glu Lys Ser Pro Glu Gln Arg Gly Arg Phe  
 945 950 955 960  
 Arg Ala Met Val Glu Leu Ala Arg Leu Asp Arg Arg Arg Pro Gly Ser  
 965 970 975  
 Ser Asp Arg Val Leu Phe Ala Arg Phe Ser Lys Thr Glu Gly Gly Ala  
 980 985 990  
 Arg Arg Ala Ser Pro Asp Gln Glu Ala Glu Asp Leu Trp Leu Ser Pro  
 995 1000 1005  
 Leu Thr Met Glu Asp Leu Val Cys Tyr Ser Phe Gln Val Ala Arg Gly  
 1010 1015 1020  
 Met Glu Phe Leu Ala Ser Arg Lys Cys Ile His Arg Asp Leu Ala Ala  
 1025 1030 1035 1040  
 Arg Asn Ile Leu Leu Ser Glu Ser Asp Val Val Lys Ile Cys Asp Phe  
 1045 1050 1055  
 Gly Leu Ala Arg Asp Ile Tyr Lys Asp Pro Asp Tyr Val Arg Lys Gly  
 1060 1065 1070  
 Ser Ala Arg Leu Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asp  
 1075 1080 1085  
 Lys Val Tyr Thr Thr Gln Ser Asp Val Trp Ser Phe Gly Val Leu Leu  
 1090 1095 1100  
 Trp Glu Ile Phe Ser Leu Gly Ala Ser Pro Tyr Pro Gly Val Gln Ile  
 1105 1110 1115 1120  
 Asn Glu Glu Phe Cys Gln Arg Val Arg Asp Gly Thr Arg Met Arg Ala  
 1125 1130 1135  
 Pro Glu Leu Ala Thr Pro Ala Ile Arg His Ile Met Leu Asn Cys Trp  
 1140 1145 1150  
 Ser Gly Asp Pro Lys Ala Arg Pro Ala Phe Ser Asp Leu Val Glu Ile  
 1155 1160 1165  
 Leu Gly Asp Leu Leu Gln Gly Arg Gly Leu Gln Glu Glu Glu Val  
 1170 1175 1180  
 Cys Met Ala Pro Arg Ser Ser Gln Ser Ser Glu Glu Gly Ser Phe Ser



1185	1190	1195	1200
Gln Val Ser Thr Met Ala Leu His Ile Ala Gln Ala Asp Ala Glu Asp	1205	1210	1215
Ser Pro Pro Ser Leu Gln Arg His Ser Leu Ala Ala Arg Tyr Tyr Asn	1220	1225	1230
Trp Val Ser Phe Pro Gly Cys Leu Ala Arg Gly Ala Glu Thr Arg Gly	1235	1240	1245
Ser Ser Arg Met Lys Thr Phe Glu Glu Phe Pro Met Thr Pro Thr Thr	1250	1255	1260
Tyr Lys Gly Ser Val Asp Asn Gln Thr Asp Ser Gly Met Val Leu Ala	1265	1270	1275
Ser Glu Glu Phe Glu Gln Ile Glu Ser Arg His Arg Gln Glu Ser Gly	1285	1290	1295
Phe Arg			

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<210> 28
<211> 1356
<212> PRT
<213> Homo sapiens
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<400> 28
Met Gln Ser Lys Val Leu Leu Ala Val Ala Leu Trp Leu Cys Val Glu
  1              5              10              15

Thr Arg Ala Ala Ser Val Gly Leu Pro Ser Val Ser Leu Asp Leu Pro
              20              25              30

Arg Leu Ser Ile Gln Lys Asp Ile Leu Thr Ile Lys Ala Asn Thr Thr
  35              40              45

Leu Gln Ile Thr Cys Arg Gly Gln Arg Asp Leu Asp Trp Leu Trp Pro
  50              55              60

Asn Asn Gln Ser Gly Ser Glu Gln Arg Val Glu Val Thr Glu Cys Ser
  65              70              75              80

Asp Gly Leu Phe Cys Lys Thr Leu Thr Ile Pro Lys Val Ile Gly Asn
              85              90              95

Asp Thr Gly Ala Tyr Lys Cys Phe Tyr Arg Glu Thr Asp Leu Ala Ser
              100              105              110

Val Ile Tyr Val Tyr Val Gln Asp Tyr Arg Ser Pro Phe Ile Ala Ser
              115              120              125

Val Ser Asp Gln His Gly Val Val Tyr Ile Thr Glu Asn Lys Asn Lys
  130              135              140

Thr Val Val Ile Pro Cys Leu Gly Ser Ile Ser Asn Leu Asn Val Ser
  145              150              155              160

Leu Cys Ala Arg Tyr Pro Glu Lys Arg Phe Val Pro Asp Gly Asn Arg

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165										170				175			
Ile	Ser	Trp	Asp	Ser	Lys	Lys	Gly	Phe	Thr	Ile	Pro	Ser	Tyr	Met	Ile		
			180					185						190			
Ser	Tyr	Ala	Gly	Met	Val	Phe	Cys	Glu	Ala	Lys	Ile	Asn	Asp	Glu	Ser		
		195					200					205					
Tyr	Gln	Ser	Ile	Met	Tyr	Ile	Val	Val	Val	Val	Gly	Tyr	Arg	Ile	Tyr		
	210					215					220						
Asp	Val	Val	Leu	Ser	Pro	Ser	His	Gly	Ile	Glu	Leu	Ser	Val	Gly	Glu		
225					230					235					240		
Lys	Leu	Val	Leu	Asn	Cys	Thr	Ala	Arg	Thr	Glu	Leu	Asn	Val	Gly	Ile		
				245					250					255			
Asp	Phe	Asn	Trp	Glu	Tyr	Pro	Ser	Ser	Lys	His	Gln	His	Lys	Lys	Leu		
			260					265					270				
Val	Asn	Arg	Asp	Leu	Lys	Thr	Gln	Ser	Gly	Ser	Glu	Met	Lys	Lys	Phe		
		275					280					285					
Leu	Ser	Thr	Leu	Thr	Ile	Asp	Gly	Val	Thr	Arg	Ser	Asp	Gln	Gly	Leu		
	290					295					300						
Tyr	Thr	Cys	Ala	Ala	Ser	Ser	Gly	Leu	Met	Thr	Lys	Lys	Asn	Ser	Thr		
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Cys Glu Arg Leu Pro Tyr Asp Ala Ser Lys Trp Glu Phe Pro Arg Asp  
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 850 855 860  
 Val Ala Val Lys Met Leu Lys Glu Gly Ala Thr His Ser Glu His Arg  
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 Ala Leu Met Ser Glu Leu Lys Ile Leu Ile His Ile Gly His His Leu  
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&lt;223&gt; Xaa equals any amino acid

&lt;220&gt;

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&lt;220&gt;

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 gactctagag gat 733

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/14925

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : C07H 21/04; C12N 15/63

US CL : 536/23.1; 435/320.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MPSRCH

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BETSHOLTZ et al. CDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumor cell lines. Nature. 24 April 1986, Vol. 320, No. 6064, pages 695-699. See entire document, especially attached gene sequence.	1-4
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Y		7-10,14,15,17

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*I* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

29 SEPTEMBER 2000

Date of mailing of the international search report

20 OCT 2000

 Name and mailing address of the ISA/US  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MINH-TAM DAVIS

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/14925**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-10, 14-15, 17, 21, SEQ ID NO. 1

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/14925

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-10, 14-15, 17 and 21, drawn to 1) a polynucleotide having a nucleotide sequence at least 95% identical to a fragment or a variant of SEQ ID NO: X, or a polynucleotide encoding a fragment or a domain or an epitope or a species homologue of SEQ ID NO: Y, 2) a vector comprising said polynucleotide, and a host cell comprising said vector, 3) a method of making said polypeptide, and 4) a method for preventing, treating, or ameliorating a medical condition, comprising administering said polynucleotide.

Group II, claim(s) 11, 12 and 16, drawn to a polypeptide fragment or domain or epitope or variant or species homologue or a secreted form of SEQ ID NO: Y.

Group III, claim(s) 13, drawn to an antibody that binds specifically to said polypeptide.

Group IV, claim 18, drawn to a method for diagnosing a pathological condition, or susceptibility to a pathological condition, by determining a mutation in said polynucleotide.

Group V, claim 19, drawn to a method for diagnosing a pathological condition, or susceptibility to a pathological condition, by determining the presence or amount of said polypeptide.

Group VI, claim 20, drawn to a method for identifying a binding partner of said polypeptide.

Group VII, claim 22, drawn to a method of identifying an activity in a biological sample.

Group VIII, claim 23, drawn to a binding partner of said polypeptide.

This application contains claims directed to more than one subgroup of the generic invention. These subgroups are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one subgroup to be searched, the appropriate additional search fees must be paid. The species are as follows:

From Groups I, or IV, anyone of the subgroup of SEQ ID NOs: 1,3,5,7,9,11,13,15,17,19,21,23.

From Groups II, III, V-VIII, anyone of the subgroup of SEQ ID NOs: 2,4,6,8,10,12,14,16,18,20,22,24.

The inventions listed as Groups I-VIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

An international stage application shall relate to one invention only or to a group of invention so linked as to form a single general inventive concept. If multiple products, processes of manufacture or uses are claimed, the first invention of the category first mentioned in the claims of the application will be considered as the main invention in the claims, see PCT article 17(3) (a) and 1.476 (c), 37 C.F.R. 1.475(d). Group I will be the main invention. After that, all other products and methods will be broken out as separate groups (see 37 CFR 1.475 (d)).

Group I, claims 1-10, 14, 15, 17 and 21 form a single inventive concept, comprising a polynucleotide and a method of making and using said polynucleotide for treating a disease.

Group II is an additional product, a polypeptide encoded by said polynucleotide, which is structurally distinct from the polynucleotide of group I.

In addition, the polynucleotide of claim 1, SEQ ID NO: 1 is known in the art (Accession NO: X03795, Betsholtz et al, Nature, 1986, vol. 320, No.6064, pages 695-699, and MPSRCH search report, pages 1-3). Therefore, the polynucleotide of claim 1 is not novel to form a special technical feature linking groups I-VIII.

Group III is an additional product, an antibody which is structurally distinct from the polynucleotide of claim 1.

Groups IV-VII, drawn to additional methods, which are distinct from the methods of group I and each others by having different objectives, and/or using different means.

Group VIII, drawn to an additional product, a binding protein, which is structurally distinct from the polynucleotide of group I.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

The subgroup polynucleotides are structurally distinct from each other.

The subgroup polypeptides are structurally distinct from each other.